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(54) Title: REMODELING AND GLYCOPEGYLATION OF FIBROBLAST GROWTH FACTOR (FGF)

(57) Abstract: The present invention relates to mutants of Fibroblast Growth Factor (FGF), particularly FGF-20 and FGF-2 1, which contain newly introduced N-linked or O-linked glycosylation site(s). The polynucleotide coding sequences for the mutants, expression cassettes comprising the coding sequences, cells expressing the mutants, and methods for producing the mutants are also disclosed. Further disclosed are pharmaceutical compositions comprising the mutants and method for using the mutants.

REMODELING AND GLYCOPEGYLATION OF FIBROBLAST GROWTH FACTOR (FGF)

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application is related to U.S. Provisional Patent Application 60/623,342, filed October 29, 2004, which is incorporated by reference in its entirety for all purposes.

BACKGROUND OF THE INVENTION

[0002] Fibroblast Growth Factors (FGFs) promote growth, proliferation, survival and differentiation of a wide variety of cells and tissue types. The prototypic fibroblast growth factors (FGFs), FGF-1 and FGF-2, were originally isolated from brain and pituitary as mitogens for fibroblasts. However, FGF-1 and FGF-2, and fibroblast growth factors generally, are widely expressed in developing and adult tissues, and have multiple biological activities including angiogenesis, mitogenesis, cellular differentiation and repair of tissue injury (*see e.g.,* Baird, A. et al., Cancer Cells 3:239-243 (1991) and Burgess, W. H. et al., Annu. Rev. Biochem. 58:575-606 (1989)).

[0003] According to the published literature, the FGF family now consists of at least twenty five members, FGF-1 to FGF-25. The 25 members of the FGF family range in molecular mass from 17 to 34 kDa and share 13-71% amino acid identity. Between vertebrate species, FGFs are highly conserved in both gene structure and amino-acid sequence.

[0004] The 25 members of the mammalian FGF family are differentially expressed in many tissues. The members are divided into subfamilies that have similar, though individually unique, patterns of expression. Some FGFs are expressed exclusively during embryonic development (for example, *Fgf3, 4, 8, 15, 17* and *19*), whereas others are expressed in embryonic and adult tissues. For example, *FGF-16*mRNA is predominantly expressed in the rat heart in adult tissues. However, in rat embryos, *FGF-16*mRNA is predominantly expressed in the brown adipose tissue (*see e.g.*, Miyake A, et al. *Biochem Biophys Res Commun* 1998, **243:**148-152).

[0005] Although most FGFs (FGFs 3-8, 10, 15, 17-19, and 21-25) have amino-terminal signal peptides and are readily secreted from cells, FGFs 9, 16 and 20 lack an obvious amino-terminal signal peptide but are nevertheless secreted (*see e.g.*, Miyamoto M, et al. *Mol Cell Biol* 1993, **13**:4251-4259). A third subset of FGFs (FGF 11-14) lack signal sequences and are thought to remain intracellular.

[0006] As noted above, the sub-family of FGF proteins comprising FGF-9, FGF-16, and FGF-20 lack a classical signal sequence, although they contain nuclear localization signals, and are secreted. These FGFs are expressed in the developing and adult nervous systems, suggesting a role in nervous system development and function (*see e.g.*, Smallwood P.M., *et al. Proc Natl Acad Sci USA* (1996) **93**:9850-9857). Indeed, a cDNA encoding FGF-20 was isolated from rat brain (*see e.g.*, U.S. Patent 6,797,695). Among FGF family members, FGF-20 is most similar to FGF-9 and FGF-16 (70 and 62% amino acid identity, respectively).

[0007] Numerous studies of human disorders as well as gene knock-out studies in mice indicate that FGFs are neurotrophic for cells of both the peripheral and central nervous system, and are important in the development of the skeletal system in mammals. A role in nervous system development and function is supported by *in situ* hybridization studies that show that FGF-20 mRNA is preferentially expressed in the substantia nigra pars compacta of the brain. Further support for a nervous system function is found in studies showing that *in vitro*, recombinant rat FGF-20 enhanced the survival of midbrain dopaminergic neurons in culture (*see e.g.,* Ohmachi S. *Biochem Biophys Res Commun* 2000, **277**:355-360).

[0008] In other studies, high levels of FGF-21 mRNA expression has been shown to occur in the liver, and human FGF-21 may play a role in the development of and recovery from liver disease. FGF-21 is also expressed in testis and thymus, and therefore may play a role in the development or recovery from disorders of testicular function or function of cells derived from the thymus (*see e.g.,* U.S. Patent No. 6,716,626).

[0009] Because of their wide ranging and potent activities, FGFs are pursued as therapeutic agents for a number of different indications, including wound healing, such as musculo-skeletal conditions, bone fractures, ligament and tissue repair, tendonitis, bursitis, etc.; skin conditions, for example, burns, cuts, lacerations, bed sores, slow healing ulcers, etc.; tissue protection, repair, and the induction of angiogenesis during myocardial infarction and ischemia, inflammatory conditions and diseases (*e.g.,* intestinal inflammation, including inflammatory bowel disease *see e.g.*,Jeffers *et al. Gastroenterology 2002;123:1151 –1162*),

in the treatment of neurological conditions such as neuro-degenerative diseases (*e.g.*, Parkinson's disease), and stroke, in the treatment of eye disease, including macular degeneration, the pathology and treatment of cancer (*see e.g.*, Jeffers, M., *et al. Cancer Research* 61, 3131-3138, April 1, (2001) and Jeffers *et al. Expert Opinion on Therapeutic Targets* (2002) 6(4):469-482) and for the treatment of diabetes. Unfortunately, the administration of therapeutic proteins such as FGF-9, FGF-18, FGF-20, and FGF-21 for the treatment of diseases and conditions can be complicated by, for example, short half life and mutagenic properties.

[0010] Poly(ethylene glycol) ("PEG") is an exemplary polymer that has been conjugated to polypeptides. The use of PEG to derivatize peptide therapeutics has been demonstrated to reduce the immunogenicity of the peptides and improve pharmacodynamics including half-life. For example, U.S. Pat. No. 4,179,337 (Davis *et al.*) concerns non-immunogenic polypeptides, such as enzymes and peptide hormones coupled to polyethylene glycol (PEG) or polypropylene glycol. Between 10 and 100 moles of polymer are used per mole polypeptide and at least 15% of the physiological activity is maintained. In addition, the clearance time in circulation is prolonged due to the increased size of the PEG-conjugate of the polypeptides in question. The methods disclosed by Davis et al. are chemical PEG-ylation methods.

[0011] The chemical modification of peptides, frequently results in an undesirable loss of peptide activity, which is attributable to the non-selective nature of the chemistries utilized to modify the peptide. For example, when the modifying group is a water-soluble peptide, *e.g.*, PEG, the principal mode of attachment of PEG, and its derivatives, to peptides is a non-specific bonding through a peptide amino acid residue. Studies of conjugates of water-soluble polymers and interleukin-2 (Fisher et al., *Br. J. Haematol.*, **82**: 654 (1992)), granulocyte colony stimulating factor (Satake-Ishikawa et al., *Cell Struct. Funct.*, **17**: 157 (1992)), tumor necrosis factor (Tsutsumi et al., *Br. J. Cancer*, **71**: 963 (1996)) and Fibroblast Growth Factor (Clark, et al., *J. Biol. Chem.*, **271**:21969 (1996)) have revealed that chemical PEGylation of these proteins decreases the in vivo receptor binding activity of the peptides.

[0012] In many chemical PEGylation methods, poly(ethylene glycol) is added in an essentially random, non-specific manner to reactive residues on a peptide backbone. For the production of therapeutic peptides, it is clearly desirable to utilize a derivitization strategy that results in the formation of a specifically labeled, readily characterizable, essentially

homogeneous product. A promising route to preparing specifically labeled peptides is through the use of enzymes, such as glycosyltransferases to append a modified sugar moiety onto a peptide.

[0013] Enzyme-based syntheses have the advantages of regioselectivity and stereoselectivity. Moreover, enzymatic syntheses are performed using unprotected substrates. Two principal classes of enzymes are used in the synthesis of carbohydrates, glycosyltransferases (*e.g.*, sialyltransferases, oligosaccharyltransferases, N-acetylglucosaminyltransferases), and glycosidases. The glycosidases are further classified as exoglycosidases (*e.g.*, β-mannosidase, β-glucosidase), and endoglycosidases (*e.g.*, Endo-A, Endo-M). Each of these classes of enzymes has been successfully used synthetically to prepare carbohydrates. For a general review, *see,* Crout *et al., Curr. Opin. Chem. Biol.* **2**: 98-111 (1998).

[0014] Glycosyltransferases modify the oligosaccharide structures on glycopeptides, producing specific products with good stereochemical and regiochemical control. Glycosyltransferases are used to prepare oligosaccharides and to modify terminal N- and O-linked carbohydrate structures, particularly on glycopeptides produced in mammalian cells. For example, the terminal oligosaccharides of glycopeptides have been completely sialylated and/or fucosylated to provide more consistent sugar structures, which improves glycopeptide pharmacodynamics and a variety of other biological properties. For example, β-1,4-galactosyltransferase was used to synthesize lactosamine, an illustration of the utility of glycosyltransferases in the synthesis of carbohydrates (*see, e.g.*, Wong *et al., J. Org. Chem.* **47**: 5416-5418 (1982)). Moreover, numerous synthetic procedures have made use of α-sialyltransferases to transfer sialic acid from cytidine-5'-monophospho-N-acetylneuraminic acid to the 3-OH or 6-OH of galactose (*see, e.g.*, Kevin *et al., Chem. Eur. J.* **2**: 1359-1362 (1996)). Fucosyltransferases are used in synthetic pathways to transfer a fucose unit from guanosine-5'-diphosphofucose to a specific hydroxyl of a saccharide acceptor. For example, Ichikawa prepared sialyl Lewis-X by a method that involves the fucosylation of sialylated lactosamine with a cloned fucosyltransferase (Ichikawa *et al., J. Am. Chem. Soc.* **114**: 9283-9298 (1992)). For a discussion of recent advances in glycoconjugate synthesis for therapeutic use *see,* Koeller *et al., Nature Biotechnology* **18**: 835-841 (2000). *See* also, U.S. Patent No. 5,876,980; 6,030,815; 5,728,554; 5,922,577; and WO/9831826.

[0015] Glycosidases can also be used to prepare saccharides. Glycosidases normally catalyze the hydrolysis of a glycosidic bond. Under appropriate conditions, however, they can be used to form this linkage. Most glycosidases used for carbohydrate synthesis are exoglycosidases; the glycosyl transfer occurs at the non-reducing terminus of the substrate. The glycosidase takes up a glycosyl donor in a glycosyl-enzyme intermediate that is either intercepted by water to give the hydrolysis product, or by an acceptor, to give a new glycoside or oligosaccharide. An exemplary pathway using an exoglycosidase is the synthesis of the core trisaccharide of all N-linked glycopeptides, including the difficult β-mannoside linkage, which was formed by the action of β-mannosidase (Singh *et al., Chem. Commun.* 993-994 (1996)).

[0016] In another exemplary application of the use of a glycosidase to form a glycosidic linkage, a mutant glycosidase has been prepared in which the normal nucleophilic amino acid within the active site is changed to a non-nucleophilic amino acid. The mutant enzymes do not hydrolyze glycosidic linkages, but can still form them. The mutant glycosidases are used to prepare oligosaccharides using an α-glycosyl fluoride donor and a glycoside acceptor molecule (Withers *et al.*, U.S. Patent No. 5,716,812). Although the mutant glycosidases are useful for forming free oligosaccharides, it has yet to be demonstrated that such enzymes are capable of appending glycosyl donors onto glycosylated or non-glycosylated peptides, nor have these enzymes been used with unactivated glycosyl donors.

[0017] Although their use is less common than that of the exoglycosidases, endoglycosidases are also utilized to prepare carbohydrates. Methods based on the use of endoglycosidases have the advantage that an oligosaccharide, rather than a monosaccharide, is transferred. Oligosaccharide fragments have been added to substrates using *endo*-β-N-acetylglucosamines such as *endo*-F, *endo*-M (Wang *et al., Tetrahedron Lett.* **37**: 1975-1978); and Haneda *et al., Carbohydr. Res.* **292**: 61-70 (1996)).

[0018] In addition to their use in preparing carbohydrates, the enzymes discussed above are applied to the synthesis of glycopeptides as well. The synthesis of a homogenous glycoform of ribonuclease B has been published (Witte K. *et al., J. Am. Chem. Soc.* **119**: 2114-2118 (1997)). The high mannose core of ribonuclease B was cleaved by treating the glycopeptide with endoglycosidase H. The cleavage occurred specifically between the two core GlcNAc residues. The tetrasaccharide sialyl Lewis X was then enzymatically rebuilt on the remaining GlcNAc anchor site on the now homogenous protein by the sequential use of β-1,4-

galactosyltransferase, α-2,3-sialyltransferase and α-1,3-fucosyltransferase V. Each enzymatically catalyzed step proceeded in excellent yield.

[0019] Methods combining both chemical and enzymatic synthetic elements are also known. For example, Yamamoto and coworkers (*Carbohydr. Res.* **305**: 415-422 (1998)) reported the chemoenzymatic synthesis of the glycopeptide, glycosylated Peptide T, using an endoglyosidase. The N-acetylglucosaminyl peptide was synthesized by purely chemical means. The peptide was subsequently enzymatically elaborated with the oligosaccharide of human transferrin glycopeptide. The saccharide portion was added to the peptide by treating it with an endo-β-N-acetylglucosaminidase. The resulting glycosylated peptide was highly stable and resistant to proteolysis when compared to the peptide T and N-acetylglucosaminyl peptide T.

[0020] The use of glycosyltransferases to modify peptide structure with reporter groups has been explored. For example, Brossmer *et al.* (U.S. Patent No. 5,405,753) discloses the formation of a fluorescent-labeled cytidine monophosphate ("CMP") derivative of sialic acid and the use of the fluorescent glycoside in an assay for sialyl transferase activity and for the fluorescent-labeling of cell surfaces, glycoproteins and gangliosides. Gross *et al.* (*Analyt. Biochem.* **186**: 127 (1990)) describe a similar assay. Bean *et al.* (U.S. Patent No. 5,432,059) discloses an assay for glycosylation deficiency disorders utilizing reglycosylation of a deficiently glycosylated protein. The deficient protein is reglycosylated with a fluorescent-labeled CMP glycoside. Each of the fluorescent sialic acid derivatives is substituted with the fluorescent moiety at either the 9-position or at the amine that is normally acetylated in sialic acid. The methods using the fluorescent sialic acid derivatives are assays for the presence of glycosyltransferases or for non-glycosylated or improperly glycosylated glycoproteins. The assays are conducted on small amounts of enzyme or glycoprotein in a sample of biological origin. The enzymatic derivatization of a glycosylated or non-glycosylated peptide on a preparative or industrial scale using a modified sialic acid was not disclosed or suggested in either of these references.

[0021] Enzymatic methods have also been used to activate glycosyl residues on a glycopeptide towards subsequent chemical elaboration. The glycosyl residues are typically activated using galactose oxidase, which converts a terminal galactose residue to the corresponding aldehyde. The aldehyde is subsequently coupled to an amine-containing

modifying group. For example, Casares *et al.* (*Nature Biotech.* **19**: 142 (2001)) have attached doxorubicin to the oxidized galactose residues of a recombinant MHCII-peptide chimera.

[0022] Glycosyl residues have also been modified to bear ketone groups. For example, Mahal and co-workers (*Science* **276**: 1125 (1997)) have prepared N-levulinoyl mannosamine ("ManLev"), which has a ketone functionality at the position normally occupied by the acetyl group in the natural substrate. Cells were treated with the ManLev, thereby incorporating a ketone group onto the cell surface. *See,* also Saxon *et al.*, *Science* **287**: 2007 (2000); Hang *et al.*, *J. Am. Chem. Soc.* **123**: 1242 (2001); Yarema *et al.*, *J. Biol. Chem.* **273**: 31168 (1998); and Charter *et al.*, *Glycobiology* **10**: 1049 (2000).

[0023] Carbohydrates are attached to glycopeptides in several ways of which N-linked to asparagine and mucin-type O-linked to serine and threonine are the most relevant for recombinant glycoprotein therapeuctics. A determining factor for initiation of glycosylation of a protein is the primary sequence context, although clearly other factors including protein region and conformation play roles. N-linked glycosylation occurs at the consensus sequence NXS/T, where X can be any amino acid but proline.

[0024] The present invention answers these needs by providing FGF mutants that contain newly introduced N-linked or O-linked glycosylation sites, providing flexibility in glycosylation and/or glycopegylation of these recombinant FGF mutants. Moreover, the invention provides an industrially practical method for the modification of N- or O-linked mutant FGF peptides with modifying groups such as water-soluble polymers, therapeutic moieties, biomolecules, and the like. Of particular interest are methods in which the modified mutant FGF has improved properties, which enhance its use as a therapeutic or diagnostic agent.

BRIEF SUMMARY OF THE INVENTION

[0025] It has now been discovered that the controlled modification of Fibroblast Growth Factor with one or more modifying groups (*e.g.*, non-glycosidic modifying groups) affords a novel FGF peptide conjugate with pharmacokinetic properties that are improved relative to the corresponding native (un-modified) FGF. Furthermore, cost effective methods for reliable and reproducible production of the FGF peptide conjugates of the invention have been discovered and developed.

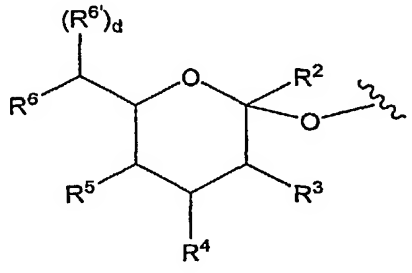
[0026] In one aspect, the invention provides an FGF conjugate comprising a FGF peptide and a glycosyl linking group—poly(ethylene glycol) cassette attached to an amino acid residue of the FGF peptide.

[0027] In an exemplary embodiment, glycconjugated FGF molecules of the invention are produced by the enzyme mediated formation of a conjugate between a glycosylated or non-glycosylated FGF peptide and an enzymatically transferable saccharyl moiety that includes a modifying group, such as a polymeric modifying group, *e.g.*, poly(ethylene glycol), within its structure. The modifying group is attached to the saccharyl moiety directly (i.e., through a single group formed by the reaction of two reactive groups) or through a linker moiety, *e.g.*, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, etc.

[0028] In one aspect, the present invention provides a conjugate between a PEG moiety, and a peptide that has an *in vivo* activity similar or otherwise analogous to art-recognized FGF. In the conjugate of the invention, the PEG moiety is covalently attached to the peptide via a glycosyl linking group or an intact glycosyl linking group. Exemplary intact glycosyl linking groups include sialic acid moieties that are derivatized with PEG.

[0029] The saccharyl moiety bearing the polymeric modifying group can be attached at any position of a glycosyl moiety of FGF. Moreover, the polymeric modifying group can be bound to a glycosyl residue at any position in the amino acid sequence of a wild type or mutant FGF peptide.

[0030] In an exemplary embodiment, the invention provides a FGF peptide that is conjugated through a glycosyl linking group to a polymeric modifying group. Exemplary FGF peptide conjugates include a glycosyl linking group having a formula selected from:



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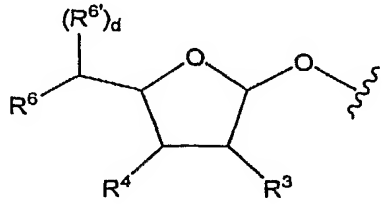
; and

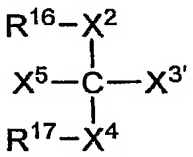


II

[0031] In Formulae I and II, R^2 is H, CH_2OR^7, $COOR^7$, COO^-M^+ or OR^7, in which R^7 represents H, substituted or unsubstituted alkyl or substituted or unsubstituted heteroalkyl. The symbols R^3, R^4, R^5, R^6 and $R^{6'}$ independently represent H, substituted or unsubstituted

alkyl, OR^8, $NHC(O)R^9$. M^+ is a metal. The index d is 0 or 1. R^8 and R^9 are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl or sialic acid. At least one of R^3, R^4, R^5, R^6 or $R^{6'}$ includes the polymeric modifying group *e.g.*, PEG. In an exemplary embodiment, R^6 and $R^{6'}$, together with the carbon to which they are attached are components of the side chain of a sialyl moiety. In a further exemplary embodiment, this side chain is functionalized with the polymeric modifying group.

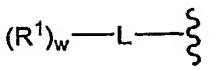
[0032] As discussed herein, the PEG of use in the conjugates of the invention can be linear or branched. An exemplary precursor of use to form the branched PEG containing peptide conjugates according to this embodiment of the invention has the formula:



(III).

The branched polymer species according to this formula are essentially pure water-soluble polymers. $X^{3'}$ is a moiety that includes an ionizable (*e.g.*, OH, COOH, H_2PO_4, HSO_3, NH_2, and salts thereof, etc.) or other reactive functional group, *e.g.*, *infra.* C is carbon. X^5, R^{16} and R^{17} are independently selected from non-reactive groups (*e.g.*, H, unsubstituted alkyl, unsubstituted heteroalkyl) and polymeric arms (*e.g.*, PEG). X^2 and X^4 are linkage fragments that are preferably essentially non-reactive under physiological conditions, which may be the same or different. An exemplary linker includes neither aromatic nor ester moieties. Alternatively, these linkages can include one or more moiety that is designed to degrade under physiologically relevant conditions, *e.g.*, esters, disulfides, etc. X^2 and X^4 join polymeric arms R^{16} and R^{17} to C. When $X^{3'}$ is reacted with a reactive functional group of complementary reactivity on a linker, sugar or linker-sugar cassette, $X^{3'}$ is converted to a component of linkage fragment X^3.

[0033] In an exemplary embodiment, the polymeric modifying group is bound to the glycosyl linking group, generally through a heteroatom on the glycosyl core (*e.g.*, N, O), through a linker, L, as shown below:



R^1 is the polymeric modifying group and L is selected from a bond and a linking group. The index w represents an integer selected from 1-6, preferably 1-3 and more preferably 1-2. Exemplary linking groups include substituted or unsubstituted alkyl, substituted or

unsubstituted heteroalkyl moieties and sialic acid. An exemplary component of the linker is an acyl moiety. Another exemplary linking group is an amino acid residue (*e.g.*, cysteine, serine, lysine, and short oligopeptides, *e.g.*, Lys-Lys, Lys-Lys-Lys, Cys-Lys, Ser-Lys, etc.)

[0034] When L is a bond, it is formed by reaction of a reactive functional group on a precursor of R^1 and a reactive functional group of complementary reactivity on a precursor of the glycosyl linking group. When L is a non-zero order linking group, L can be in place on the glycosyl moiety prior to reaction with the R^1 precursor. Alternatively, the precursors of R^1 and L can be incorporated into a preformed cassette that is subsequently attached to the glycosyl moiety. As set forth herein, the selection and preparation of precursors with appropriate reactive functional groups is within the ability of those skilled in the art. Moreover, coupling of the precursors proceeds by chemistry that is well understood in the art.

[0035] In another aspect, the present invention provides an isolated nucleic acid comprising a polynucleotide sequence encoding a mutant Fibroblast Growth Factor. The mutant Fibroblast Growth Factor comprises one or more N-linked or O-linked glycosylation site that is not present in wild-type Fibroblast Growth Factor. In some embodiments, the nucleic acid encoding the mutant FGF-20 has a corresponding wild-type sequence that encodes a wild-type Fibroblast Growth Factor that has the amino acid sequence of SEQ ID NO:1. In some preferred embodiments, the mutant Fibroblast Growth Factor includes at least one amino acid sequence selected from SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, and 112-145. In some other embodiments, the nucleic acid encoding the mutant FGF-21 has a corresponding wild-type sequence that encodes a wild-type Fibroblast Growth Factor that has the amino acid sequence of SEQ ID NO:146. In some preferred embodiments, the mutant Fibroblast Growth Factor includes at least one amino acid sequence selected from SEQ ID NOs: 161-214, 220-320, and 323-360.

[0036] In another aspect, the present invention provides an expression cassette or a cell that comprises a nucleic acid, *e.g.*, an isolated nucleic acid, including a polynucleotide sequence encoding a mutant Fibroblast Growth Factor. The mutant Fibroblast Growth Factor includes one or more N-linked or O-linked glycosylation site that is not present in the wild-type Fibroblast Growth Factor.

[0037] In another aspect, the present invention provides a mutant Fibroblast Growth Factor, that includes one or more N-linked or O-linked glycosylation site that is not present in the wild-type Fibroblast Growth Factor. In some embodiments, the wild-type Fibroblast Growth

Factor has the amino acid sequence of SEQ ID NO:1. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, and 112-145. In some other embodiments, the wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:146. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 161-214, 220-320, and 323-360.

[0038] In another aspect, the present invention provides a method for making a mutant Fibroblast Growth Factor that includes an N-linked or O-linked glycosylation site that is not present in the wild-type Fibroblast Growth Factor. This method includes the steps of recombinantly producing the mutant Fibroblast Growth Factor, and glycosylating the mutant Fibroblast Growth Factor at the new glycosylation site. In some embodiments, the wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:1. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, and 112-145. In some other embodiments, the wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:146. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 161-214, 220-320, and 323-360.

[0039] In still a further aspect, the present invention provides a pharmaceutical composition having a therapeutically effective amount of a mutant Fibroblast Growth Factor that includes an N-linked or O-linked glycosylation site not present in the wild-type Fibroblast Growth Factor. In some embodiments, the wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:1. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, and 112-145. In some other embodiments, the wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:146. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 161-214, 220-320, and 323-360.

[0040] In each of the aspects described above, the mutant Fibroblast Growth Factor is optionally conjugated to one or more modifying groups, preferably via glycoconjugation, giving rise to a glycosyl linking group between the glycosylation site and the modifying group. An exemplary modifying group is poly(ethylene glycol).

BRIEF DESCRIPTION OF THE DRAWINGS

[0041] **FIG. 1A** displays results of an SDS-PAGE analysis of human FGF-20 induction at varied temperature, time, vector and E.Coli strains: lanes 1 and 14: molecular weight marker (sized in kDa), (induction temperature); lanes 2-9 and 15-18: 37°C, lanes 10-13 and 19-22: 20°C. Strain used: lanes 2-4 and 6-8 and 10-12, W3110; lanes 5, 9, and 13 BL21(DE3); lanes 15-17 and 19-21, E.Coli$_{(trxb, gor, supp)}$; lanes 18 and 22, E.Coli $_{(trxb, gor, supp)}$(DE3). Vector used: lanes 2, 6, 10, 15, 19 use vector #1; lanes 3, 7, 11, 16, 20 use vector #2; lanes 4, 8, 12, 17, 21 use vector #3; lanes 5, 9, 13, 18 and 22 use vector #4.

[0042] **FIG. 1B** displays results of an SDS-PAGE analysis of human FGF-20 solubility at varied temperature and E.Coli strains, lane 1:molecular weight marker (sized in kDa). Even numbers represent pellet and odd numbers represent supernatant. Induction temperatures used: lanes 2-3: 20°C; lanes 4-5: 30°C; lanes 6-7: 37°C; lanes 8-9: 37°C. Strain used: lanes 6-7, BL21(DE3); lanes 2-5 and 8-9, E.Coli $_{(trxb, gor, supp)}$(DE3). Vector #4 was employed.

[0043] **FIG. 1C** displays results of an SDS-PAGE analysis of human FGF-21 induction at varied temperature, time, vector and E.Coli strains: lane 1 and 15: molecular weight marker (sized in kDa), lane 2 no induction; (induction temperature); lanes 3-10 and 16-20: 37°C, lanes 11-14 and 21-23: 20°C. Strains used: lanes 3-5 and 7-9 and 11-13, W3110; lanes 6, 10, and 14 BL21(DE3); lanes 16-19 and 21-23, E.Coli $_{(trxb, gor, supp)}$; lane 20, E.Coli $_{(trxb, gor, supp)}$(DE3). Vectors used: lanes 3, 7, 11, 17, 21 are vector #1; lanes 4, 8, 12, 18, 22 are vector #2; lanes 5, 9, 13, 19 and 23 are vector #3; lanes 6, 10, 14, and 20 are vector #4.

[0044] **FIG. 1D** displays results of an SDS-PAGE analysis of human FGF-21 solubility at varied temperature and E.Coli strains, lane 1+1b:molecular weight marker (sized in kDa). Even numbers represent pellet and odd numbers represent supernatant. Induction temperature used: lanes 2-3 and 6-7: 37°C; lanes 4-5 and 8-9: 20°C; lanes 11-12: 18°C. Strain used: lanes 2-5, W3110; lanes 6-12, E.Coli $_{(trxb, gor, supp)}$. Vector #3 was employed.

[0045] **FIG. 2** is a table providing exemplary sialyltransferases of use in forming the glycoconjugates of the invention, *e.g.*, to glycoPEGylate peptides with a modified sialic acid.

DETAILED DESCRIPTION OF THE INVENTION AND THE PREFERRED EMBODIMENTS

Abbreviations

[0046] PEG, poly(ethyleneglycol); PPG, poly(propyleneglycol); Ara, arabinosyl; Fru, fructosyl; Fuc, fucosyl; Gal, galactosyl; GalNAc, N-acetylgalactosaminyl; Glc, glucosyl; GlcNAc, N-acetylglucosaminyl; Man, mannosyl; ManAc, mannosaminyl acetate; Xyl, xylosyl; NeuAc, sialyl or N-acetylneuraminyl; Sia, sialyl or N-acetylneuraminyl; and derivatives and analogues thereof.

Definitions

[0047] Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry and nucleic acid chemistry and hybridization are those well known and commonly employed in the art. Standard techniques are used for nucleic acid and peptide synthesis. The techniques and procedures are generally performed according to conventional methods in the art and various general references (*see generally*, Sambrook *et al.* MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference), which are provided throughout this document. The nomenclature used herein and the laboratory procedures in analytical chemistry, and organic synthetic described below are those well known and commonly employed in the art. Standard techniques, or modifications thereof, are used for chemical syntheses and chemical analyses.

[0048] The term "nucleic acid" or "polynucleotide" refers to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-

base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* **19**:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* **260**:2605-2608 (1985); and Rossolini *et al.*, *Mol. Cell. Probes* **8**:91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene.

[0049] The term "gene" means the segment of DNA involved in producing a polypeptide chain. It may include regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

[0050] The term "isolated," when applied to a nucleic acid or protein, denotes that the nucleic acid or protein is essentially free of other cellular components with which it is associated in the natural state. It is preferably in a homogeneous state although it can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein that is the predominant species present in a preparation is substantially purified. In particular, an isolated gene is separated from open reading frames that flank the gene and encode a protein other than the gene of interest. The term "purified" denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure.

[0051] The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid analogs described in the following patent application can be incorporated into the FGF peptide conjugates and mutant FGF sequences of the invention: U.S. Pat. App. Nos. 11/094677 (filed March 29, 2005); 11/094676 (filed March 29, 2005); 11/093798 (filed March 29, 2005); 11/093797 (filed March 29, 2005); 11/093597 (filed March 29, 2005); 10/965218 (filed Oct. 13, 2004);

11/093797 (filed March 29, 2005); 11/009635 (filed Dec. 10, 2004); 11/016348 (filed Dec. 16, 2004); 10/825867 (filed Apr. 16, 2004); 10/826919 (filed Apr. 16, 2004); and 10/686944 (now U.S. Pat. No 6,927,042, issued Aug. 9, 2005). The methods described in these applications can also be used to produce the the FGF peptide conjugates and mutant FGF sequences of the invention. "Amino acid mimetics" refers to chemical compounds having a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

[0052] There are various known methods in the art that permit the incorporation of an unnatural amino acid derivative or analog into a polypeptide chain in a site-specific manner, *see, e.g.*, WO 02/086075.

[0053] Amino acids may be referred to herein by either the commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0054] "Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, "conservatively modified variants" refers to those nucleic acids that encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein that encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid that encodes a polypeptide is implicit in each described sequence.

[0055] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which

alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

[0056] The following eight groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Glycine (G);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);
7) Serine (S), Threonine (T); and
8) Cysteine (C), Methionine (M)

(*see, e.g.*, Creighton, *Proteins* (1984)).

[0057] Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0058] In the present application, amino acid residues are numbered according to their relative positions from the most N-terminal residue, which is numbered 1, in an unmodified wild-type polypeptide sequence.

[0059] "Proximate to a proline residue," as used herein refers to an amino acid that is less than about 10 amino acids removed from a proline residue, preferably, less than about 9, 8, 7, 6 or 5 amino acids removed from a proline residue, more preferably, less than about 4, 3, 2 or 1 residues removed from a proline residue. The amino acid "proximate a proline residue" may be on the C- or N-terminal side of the proline residue.

[0060] "Polypeptide," "peptide," and "protein" are used interchangeably herein to refer to a polymer in which the monomers are amino acids and are joined together through amide

bonds, alternatively referred to as a polypeptide. Additionally, unnatural amino acids, for example, β-alanine, phenylglycine and homoarginine are also included. Amino acids that are not gene-encoded may also be used in the present invention. Furthermore, amino acids that have been modified to include reactive groups, glycosylation sites, polymers, therapeutic moieties, biomolecules and the like may also be used in the invention. All of the amino acids used in the present invention may be either the D - or L -isomer. The L -isomer is generally preferred. In addition, other peptidomimetics are also useful in the present invention. As used herein, "peptide" refers to both glycosylated and unglycosylated peptides. Also included are peptides that are incompletely glycosylated by a system that expresses the peptide. For a general review, *see,* Spatola, A. F., in CHEMISTRY AND BIOCHEMISTRY OF AMINO ACIDS, PEPTIDES AND PROTEINS, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983).

[0061] The term "peptide conjugate," refers to species of the invention in which a peptide is conjugated with a modified sugar as set forth herein.

[0062] The term "FGF" or "Fibroblast Growth Factor" refers to any of the family of twenty-five known wild-type peptides. The term also refers to amino acid sequences with the same, fewer or additional amino acids as compared to the wild-type sequence. Additional amino acids, which can be natural or unnatural, can be inserted into the beginning, middle, or end of the amino acid sequence.

[0063] The term "mutating" or "mutation," as used in the context of introducing additional N- or O-linked glycosylation site(s) into a wild-type Fibroblast Growth Factor, refers to the deletion, insertion, or substitution of any nucleotide or amino acid residue, by chemical, enzymatic, or any other means, in a polynucleotide sequence encoding a wild-type Fibroblast Growth Factor or the amino acid sequence of a wild-type Fibroblast Growth Factor, respectively, such that the amino acid sequence of the resulting Fibroblast Growth Factor comprises at least one N- or O-linked glycosylation site that does not exist in the corresponding wild-type Fibroblast Growth Factor. In the case of amino acid substitution, both conservative and non-conservative substitutions may be used to create a FGF mutant that contains a new N- or O-linked glycosylation site.

[0064] The site for a mutation introducing a new N- or O-linked glycosylation site may be located anywhere in the polypeptide. Exemplary amino acid sequences for Fibroblast Growth Factor mutants are depicted in SEQ ID NOs: 9-14, 18-22, 23-45, 48-65, 69-109, 112-

145, 161-214, 220-320, and 323-360. A "mutant Fibroblast Growth Factor" of this invention thus comprises at least one amino acid substitution, insertion, or mutated amino acid residue. On the other hand, the wild-type Fibroblast Growth Factor whose coding sequence is modified to generate a mutant Fibroblast Growth Factor can be referred to in this application as "the corresponding wild-type Fibroblast Growth Factor", or simply "wild-type peptide". For example, SEQ ID NO:1 is the amino acid sequence of the corresponding wild-type Fibroblast Growth Factor-20 for mutant Fibroblast Growth Factors having the amino acid sequences of SEQ ID NOs: 9-14, 18-22, 23-45, 48-65, 69-109, and 112-145. Likewise, SEQ ID NO:146 is the amino acid sequence of the corresponding wild-type Fibroblast Growth Factor-21 for mutant Fibroblast Growth Factors having the amino acid sequences of SEQ ID NOs: 161-214, 220-320, and 323-360.

[0065] The term "effective amount," or "an amount effective to" or a "therapeutically effective amount" or any grammatically equivalent term means the amount that produces therapeutic effects for which a substance is administered. The effects include the prevention, correction, or inhibition of progression of the symptoms of a disease/condition and related complications to any detectable extent. The exact amount will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques (*see, e.g.*, Lieberman, *Pharmaceutical Dosage Forms* (vols. 1-3, 1992); Lloyd, *The Art, Science and Technology of Pharmaceutical Compounding* (1999); and Pickar, *Dosage Calculations* (1999)).

[0066] As used herein, the term "modified sugar," refers to a naturally- or non-naturally-occurring carbohydrate that is enzymatically added onto an amino acid or a glycosyl residue of a peptide in a process of the invention. The modified sugar is selected from a number of enzyme substrates including, but not limited to sugar nucleotides (mono-, di-, and tri-phosphates), activated sugars (*e.g.*, glycosyl halides, glycosyl mesylates) and sugars that are neither activated nor nucleotides. The "modified sugar" is covalently functionalized with a "modifying group." Useful modifying groups include, but are not limited to, water-soluble polymers (PEG moieties), therapeutic moieties, diagnostic moieties, biomolecules and the like. The modifying group is preferably not a naturally occurring, or an unmodified carbohydrate. The locus of functionalization with the modifying group is selected such that it does not prevent the "modified sugar" from being added enzymatically to a peptide.

[0067] The term "water-soluble" refers to moieties that have some detectable degree of solubility in water. Methods to detect and/or quantify water solubility are well known in the art. Exemplary water-soluble polymers include peptides, saccharides, poly(ethers), poly(amines), poly(carboxylic acids) and the like. Peptides can have mixed sequences of be composed of a single amino acid, *e.g.*, poly(lysine). An exemplary polysaccharide is poly(sialic acid). An exemplary poly(ether) is poly(ethylene glycol), *e.g.*, m-PEG. Poly(ethylene imine) is an exemplary polyamine, and poly(acrylic) acid is a representative poly(carboxylic acid).

[0068] The polymer backbone of the water-soluble polymer can be poly(ethylene glycol) (i.e. PEG). However, it should be understood that other related polymers are also suitable for use in the practice of this invention and that the use of the term PEG or poly(ethylene glycol) is intended to be inclusive and not exclusive in this respect. The term PEG includes poly(ethylene glycol) in any of its forms, including alkoxy PEG, difunctional PEG, multiarmed PEG, forked PEG, branched PEG, pendent PEG (i.e. PEG or related polymers having one or more functional groups pendent to the polymer backbone), or PEG with degradable linkages therein.

[0069] The polymer backbone can be linear or branched. Branched polymer backbones are generally known in the art. Typically, a branched polymer has a central branch core moiety and a plurality of linear polymer chains linked to the central branch core. PEG is commonly used in branched forms that can be prepared by addition of ethylene oxide to various polyols, such as glycerol, pentaerythritol and sorbitol. The central branch moiety can also be derived from several amino acids, such as lysine. The branched poly(ethylene glycol) can be represented in general form as $R(\text{-PEG-OX})_m$ in which R represents the core moiety, such as glycerol or pentaerythritol, X represents a capping group or an end group, and m represents the number of arms. Multi-armed PEG molecules, such as those described in U.S. Pat. No. 5,932,462, which is incorporated by reference herein in its entirety, can also be used as the polymer backbone.

[0070] Many other polymers are also suitable for the invention. Polymer backbones that are non-peptidic and water-soluble, with from 2 to about 300 termini, are particularly useful in the invention. Examples of suitable polymers include, but are not limited to, other poly(alkylene glycols), such as poly(propylene glycol) ("PPG"), copolymers of ethylene glycol and propylene glycol and the like, poly(oxyethylated polyol), poly(olefinic alcohol),

poly(vinylpyrrolidone), poly(hydroxypropylmethacrylamide), poly(α-hydroxy acid), poly(vinyl alcohol), polyphosphazene, polyoxazoline, poly(N-acryloylmorpholine), such as described in U.S. Pat. No. 5,629,384, which is incorporated by reference herein in its entirety, and copolymers, terpolymers, and mixtures thereof. Although the molecular weight of each chain of the polymer backbone can vary, it is typically in the range of from about 100 Da to about 100,000 Da, often from about 6,000 Da to about 80,000 Da.

[0071] The term "sialic acid" or "sialyl" refers to any member of a family of nine-carbon carboxylated sugars. The most common member of the sialic acid family is N-acetyl-neuraminic acid (2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactononulopyranos-1-onic acid (often abbreviated as Neu5Ac, NeuAc, or NANA). A second member of the family is N-glycolyl-neuraminic acid (Neu5Gc or NeuGc), in which the N-acetyl group of NeuAc is hydroxylated. A third sialic acid family member is 2-keto-3-deoxy-nonulosonic acid (KDN) (Nadano *et al.* (1986) *J. Biol. Chem.* **261**: 11550-11557; Kanamori *et al.*, *J. Biol. Chem.* **265**: 21811-21819 (1990)). Also included are 9-substituted sialic acids such as a 9-O-C_1-C_6 acyl-Neu5Ac like 9-O-lactyl-Neu5Ac or 9-O-acetyl-Neu5Ac, 9-deoxy-9-fluoro-Neu5Ac and 9-azido-9-deoxy-Neu5Ac. For review of the sialic acid family, *see, e.g.*, Varki, *Glycobiology* **2**: 25-40 (1992); *Sialic Acids: Chemistry, Metabolism and Function*, R. Schauer, Ed. (Springer-Verlag, New York (1992)). The synthesis and use of sialic acid compounds in a sialylation procedure is disclosed in international application WO 92/16640, published October 1, 1992.

[0072] The "area under the curve" or "AUC", as used herein in the context of administering a peptide drug to a patient, is defined as total area under the curve that describes the concentration of drug in systemic circulation in the patient as a function of time from zero to infinity.

[0073] The term "half-life" or "t½", as used herein in the context of administering a peptide drug to a patient, is defined as the time required for plasma concentration of a drug in a patient to be reduced by one half. There may be more than one half-life associated with the peptide drug depending on multiple clearance mechanisms, redistribution, and other mechanisms well known in the art. Usually, alpha and beta half-lives are defined such that the alpha phase is associated with redistribution, and the beta phase is associated with clearance. However, with protein drugs that are, for the most part, confined to the bloodstream, there can be at least two clearance half-lives. For some glycosylated peptides, rapid beta phase clearance may be mediated via receptors on macrophages, or endothelial

cells that recognize terminal galactose, N-acetylgalactosamine, N-acetylglucosamine, mannose, or fucose. Slower beta phase clearance may occur via renal glomerular filtration for molecules with an effective radius < 2 nm (approximately 68 kD) and/or specific or non-specific uptake and metabolism in tissues. GlycoPEGylation may cap terminal sugars (*e.g.*, galactose or N-acetylgalactosamine) and thereby block rapid alpha phase clearance via receptors that recognize these sugars. It may also confer a larger effective radius and thereby decrease the volume of distribution and tissue uptake, thereby prolonging the late beta phase. Thus, the precise impact of glycoPEGylation on alpha phase and beta phase half-lives will vary depending upon the size, state of glycosylation, and other parameters, as is well known in the art. Further explanation of "half-life" is found in Pharmaceutical Biotechnology (1997, DFA Crommelin and RD Sindelar, eds., Harwood Publishers, Amsterdam, pp 101 – 120).

[0074] The term "glycoconjugation," as used herein, refers to the enzymatically mediated conjugation of a modified sugar species to an amino acid or glycosyl residue of a polypeptide, *e.g.*, a mutant Fibroblast Growth Factor of the present invention. A subgenus of "glycoconjugation" is "glyco-PEGylation," in which the modifying group of the modified sugar is poly(ethylene glycol), an alkyl derivative of PEG (*e.g.*, m-PEG) or a reactive derivative of PEG (*e.g.*, H_2N-PEG, HOOC-PEG) thereof.

[0075] The terms "large-scale" and "industrial-scale" are used interchangeably and refer to a reaction cycle that produces at least about 250 mg, preferably at least about 500 mg, and more preferably at least about 1 gram of glycoconjugate at the completion of a single reaction cycle.

[0076] The term, "glycosyl linking group," as used herein refers to a glycosyl residue to which a modifying group (*e.g.*, PEG moiety, therapeutic moiety, biomolecule) is covalently attached; the glycosyl linking group joins the modifying group to the remainder of the conjugate. In the methods of the invention, the "glycosyl linking group" becomes covalently attached to a glycosylated or unglycosylated peptide, thereby linking the agent to an amino acid and/or glycosyl residue on the peptide. A "glycosyl linking group" is generally derived from a "modified sugar" by the enzymatic attachment of the "modified sugar" to an amino acid and/or glycosyl residue of the peptide. The glycosyl linking group can be a saccharide-derived structure that is degraded during formation of modifying group-modified sugar cassette (*e.g.*, oxidation→Schiff base formation→reduction), or the glycosyl linking group

may be intact. An "intact glycosyl linking group" refers to a linking group that is derived from a glycosyl moiety in which the saccharide monomer that links the modifying group and to the remainder of the conjugate is not degraded, *e.g.*, oxidized, *e.g.*, by sodium metaperiodate. "Intact glycosyl linking groups" of the invention may be derived from a naturally occurring oligosaccharide by addition of glycosyl unit(s) or removal of one or more glycosyl unit from a parent saccharide structure.

[0077] The term, "non-glycosidic modifying group", as used herein, refers to modifying groups which do not include a naturally occurring sugar linked directly to the glycosyl linking group.

[0078] As used herein, "a radioactive agent" includes any radioisotope that is effective in diagnosing or destroying a tumor. Examples include, but are not limited to, indium-111, cobalt-60. Additionally, naturally occurring radioactive elements such as uranium, radium, and thorium, which typically represent mixtures of radioisotopes, are suitable examples of a radioactive agent. The metal ions are typically chelated with an organic chelating moiety.

[0079] Many useful chelating groups, crown ethers, cryptands and the like are known in the art and can be incorporated into the compounds of the invention (*e.g.*, EDTA, DTPA, DOTA, NTA, HDTA, *etc.* and their phosphonate analogs such as DTPP, EDTP, HDTP, NTP, *etc*). *See*, for example, Pitt *et al.*, "The Design of Chelating Agents for the Treatment of Iron Overload," In, INORGANIC CHEMISTRY IN BIOLOGY AND MEDICINE; Martell, Ed.; American Chemical Society, Washington, D.C., 1980, pp. 279-312; Lindoy, THE CHEMISTRY OF MACROCYCLIC LIGAND COMPLEXES; Cambridge University Press, Cambridge,1989; Dugas, BIOORGANIC CHEMISTRY; Springer-Verlag, New York, 1989, and references contained therein.

[0080] Additionally, a manifold of routes allowing the attachment of chelating agents, crown ethers and cyclodextrins to other molecules is available to those of skill in the art. *See*, for example, Meares *et al.*, "Properties of In Vivo Chelate-Tagged Proteins and Polypeptides." In, MODIFICATION OF PROTEINS: FOOD, NUTRITIONAL, AND PHARMACOLOGICAL ASPECTS;" Feeney, *et al.*, Eds., American Chemical Society, Washington, D.C., 1982, pp. 370-387; Kasina *et al.*, *Bioconjugate Chem.*, **9:** 108-117 (1998); Song *et al.*, *Bioconjugate Chem.*, **8:** 249-255 (1997).

[0081] As used herein, "pharmaceutically acceptable carrier" includes any material, which when combined with the conjugate retains the conjugates' activity and is non-reactive with

the subject's immune systems. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Other carriers may also include sterile solutions, tablets including coated tablets and capsules. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well known conventional methods.

[0082] As used herein, "administering," means oral administration, inhalation, administration as a suppository, topical contact, intravenous, intraperitoneal, intramuscular, intralesional, intranasal or subcutaneous administration, or the implantation of a slow-release device *e.g.*, a mini-osmotic pump, to the subject. Adminsitration is by any route including parenteral, and transmucosal (*e.g.*, oral, nasal, vaginal, rectal, or transdermal). Parenteral administration includes, *e.g.*, intravenous, intramuscular, intra-arteriole, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial. Moreover, where injection is to treat a tumor, *e.g.*, induce apoptosis, administration may be directly to the tumor and/or into tissues surrounding the tumor. Other modes of delivery include, but are not limited to, the use of liposomal formulations, intravenous infusion, transdermal patches, etc.

[0083] The term "ameliorating" or "ameliorate" refers to any indicia of success in the treatment of a pathology or condition, including any objective or subjective parameter such as abatement, remission or diminishing of symptoms or an improvement in a patient's physical or mental well-being. Amelioration of symptoms can be based on objective or subjective parameters; including the results of a physical examination and/or a psychiatric evaluation.

[0084] The term "therapy" refers to "treating" or "treatment" of a disease or condition including preventing the disease or condition from occurring in an animal that may be predisposed to the disease but does not yet experience or exhibit symptoms of the disease (prophylactic treatment), inhibiting the disease (slowing or arresting its development), providing relief from the symptoms or side-effects of the disease (including palliative treatment), and relieving the disease (causing regression of the disease).

[0085] The term "isolated" refers to a material that is substantially or essentially free from components, which are used to produce the material. For peptide conjugates of the invention,

the term "isolated" refers to material that is substantially or essentially free from components, which normally accompany the material in the mixture used to prepare the peptide conjugate. "Isolated" and "pure" are used interchangeably. Typically, isolated peptide conjugates of the invention have a level of purity preferably expressed as a range. The lower end of the range of purity for the peptide conjugates is about 60%, about 70% or about 80% and the upper end of the range of purity is about 70%, about 80%, about 90% or more than about 90%.

[0086] When the peptide conjugates are more than about 90% pure, their purities are also preferably expressed as a range. The lower end of the range of purity is about 90%, about 92%, about 94%, about 96% or about 98%. The upper end of the range of purity is about 92%, about 94%, about 96%, about 98% or about 100% purity.

[0087] Purity is determined by any art-recognized method of analysis (*e.g.*, band intensity on a silver stained gel, polyacrylamide gel electrophoresis, HPLC, or a similar means).

[0088] "Essentially each member of the population," as used herein, describes a characteristic of a population of peptide conjugates of the invention in which a selected percentage of the modified sugars added to a peptide are added to multiple, identical acceptor sites on the peptide. "Essentially each member of the population" speaks to the "homogeneity" of the sites on the peptide conjugated to a modified sugar and refers to conjugates of the invention, which are at least about 80%, preferably at least about 90% and more preferably at least about 95% homogenous.

[0089] "Homogeneity," refers to the structural consistency across a population of acceptor moieties to which the modified sugars are conjugated. Thus, in a peptide conjugate of the invention in which each modified sugar moiety is conjugated to an acceptor site having the same structure as the acceptor site to which every other modified sugar is conjugated, the peptide conjugate is said to be about 100% homogeneous. Homogeneity is typically expressed as a range. The lower end of the range of homogeneity for the peptide conjugates is about 60%, about 70% or about 80% and the upper end of the range of purity is about 70%, about 80%, about 90% or more than about 90%.

[0090] When the peptide conjugates are more than or equal to about 90% homogeneous, their homogeneity is also preferably expressed as a range. The lower end of the range of homogeneity is about 90%, about 92%, about 94%, about 96% or about 98%. The upper end of the range of purity is about 92%, about 94%, about 96%, about 98% or about 100% homogeneity. The purity of the peptide conjugates is typically determined by one or more

methods known to those of skill in the art, *e.g.*, liquid chromatography-mass spectrometry (LC-MS), matrix assisted laser desorption mass time of flight spectrometry (MALDITOF), capillary electrophoresis, and the like.

[0091] "Substantially uniform glycoform" or a "substantially uniform glycosylation pattern," when referring to a glycopeptide species, refers to the percentage of acceptor moieties that are glycosylated by the glycosyltransferase of interest (*e.g.*, fucosyltransferase). For example, in the case of a α1,2 fucosyltransferase, a substantially uniform fucosylation pattern exists if substantially all (as defined below) of the Galβ1,4-GlcNAc-R and sialylated analogues thereof are fucosylated in a peptide conjugate of the invention. It will be understood by one of skill in the art, that the starting material may contain glycosylated acceptor moieties (*e.g.*, fucosylated Galβ1,4-GlcNAc-R moieties). Thus, the calculated percent glycosylation will include acceptor moieties that are glycosylated by the methods of the invention, as well as those acceptor moieties already glycosylated in the starting material.

[0092] The term "substantially" in the above definitions of "substantially uniform" generally means at least about 40%, at least about 70%, at least about 80%, or more preferably at least about 90%, and still more preferably at least about 95% of the acceptor moieties for a particular glycosyltransferase are glycosylated.

[0093] Where substituent groups are specified by their conventional chemical formulae, written from left to right, they equally encompass the chemically identical substituents, which would result from writing the structure from right to left, *e.g.*, $-CH_2O-$ is intended to also recite $-OCH_2-$.

[0094] The term "alkyl," by itself or as part of another substituent means, unless otherwise stated, a straight or branched chain, or cyclic hydrocarbon radical, or combination thereof, which may be fully saturated, mono- or polyunsaturated and can include di- and multivalent radicals, having the number of carbon atoms designated (*i.e.* C_1-C_{10} means one to ten carbons). Examples of saturated hydrocarbon radicals include, but are not limited to, groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, isobutyl, sec-butyl, cyclohexyl, (cyclohexyl)methyl, cyclopropylmethyl, homologs and isomers of, for example, n-pentyl, n-hexyl, n-heptyl, n-octyl, and the like. An unsaturated alkyl group is one having one or more double bonds or triple bonds. Examples of unsaturated alkyl groups include, but are not limited to, vinyl, 2-propenyl, crotyl, 2-isopentenyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl), ethynyl, 1- and 3-propynyl, 3-butynyl, and the higher homologs and

isomers. The term "alkyl," unless otherwise noted, is also meant to include those derivatives of alkyl defined in more detail below, such as "heteroalkyl." Alkyl groups that are limited to hydrocarbon groups are termed "homoalkyl".

[0095] The term "alkylene" by itself or as part of another substituent means a divalent radical derived from an alkane, as exemplified, but not limited, by $-CH_2CH_2CH_2CH_2-$, and further includes those groups described below as "heteroalkylene." Typically, an alkyl (or alkylene) group will have from 1 to 24 carbon atoms, with those groups having 10 or fewer carbon atoms being preferred in the present invention. A "lower alkyl" or "lower alkylene" is a shorter chain alkyl or alkylene group, generally having eight or fewer carbon atoms.

[0096] The terms "alkoxy," "alkylamino" and "alkylthio" (or thioalkoxy) are used in their conventional sense, and refer to those alkyl groups attached to the remainder of the molecule via an oxygen atom, an amino group, or a sulfur atom, respectively.

[0097] The term "heteroalkyl," by itself or in combination with another term, means, unless otherwise stated, a stable straight or branched chain, or cyclic hydrocarbon radical, or combinations thereof, consisting of the stated number of carbon atoms and at least one heteroatom selected from the group consisting of O, N, Si and S, and wherein the nitrogen and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. The heteroatom(s) O, N and S and Si may be placed at any interior position of the heteroalkyl group or at the position at which the alkyl group is attached to the remainder of the molecule. Examples include, but are not limited to, $-CH_2-CH_2-O-CH_3$, $-CH_2-CH_2-NH-CH_3$, $-CH_2-CH_2-N(CH_3)-CH_3$, $-CH_2-S-CH_2-CH_3$, $-CH_2-CH_2,-S(O)-CH_3$, $-CH_2-CH_2-S(O)_2-CH_3$, $-CH=CH-O-CH_3$, $-Si(CH_3)_3$, $-CH_2-CH=N-OCH_3$, and $-CH=CH-N(CH_3)-CH_3$. Up to two heteroatoms may be consecutive, such as, for example, $-CH_2-NH-OCH_3$ and $-CH_2-O-Si(CH_3)_3$. Similarly, the term "heteroalkylene" by itself or as part of another substituent means a divalent radical derived from heteroalkyl, as exemplified, but not limited by, $-CH_2-CH_2-S-CH_2-CH_2-$ and $-CH_2-S-CH_2-CH_2-NH-CH_2-$. For heteroalkylene groups, heteroatoms can also occupy either or both of the chain termini (*e.g.*, alkyleneoxy, alkylenedioxy, alkyleneamino, alkylenediamino, and the like). Still further, for alkylene and heteroalkylene linking groups, no orientation of the linking group is implied by the direction in which the formula of the linking group is written. For example, the formula $-C(O)_2R'-$ represents both $-C(O)_2R'-$ and $-R'C(O)_2-$.

[0098] The terms "cycloalkyl" and "heterocycloalkyl", by themselves or in combination with other terms, represent, unless otherwise stated, cyclic versions of "alkyl" and "heteroalkyl", respectively. Additionally, for heterocycloalkyl, a heteroatom can occupy the position at which the heterocycle is attached to the remainder of the molecule. Examples of cycloalkyl include, but are not limited to, cyclopentyl, cyclohexyl, 1-cyclohexenyl, 3-cyclohexenyl, cycloheptyl, and the like. Examples of heterocycloalkyl include, but are not limited to, 1 –(1,2,5,6-tetrahydropyridyl), 1-piperidinyl, 2-piperidinyl, 3-piperidinyl, 4-morpholinyl, 3-morpholinyl, tetrahydrofuran-2-yl, tetrahydrofuran-3-yl, tetrahydrothien-2-yl, tetrahydrothien-3-yl, 1 –piperazinyl, 2-piperazinyl, and the like.

[0099] The terms "halo" or "halogen," by themselves or as part of another substituent, mean, unless otherwise stated, a fluorine, chlorine, bromine, or iodine atom. Additionally, terms such as "haloalkyl," are meant to include monohaloalkyl and polyhaloalkyl. For example, the term "halo(C_1-C_4)alkyl" is mean to include, but not be limited to, trifluoromethyl, 2,2,2-trifluoroethyl, 4-chlorobutyl, 3-bromopropyl, and the like.

[0100] The term "aryl" means, unless otherwise stated, a polyunsaturated, aromatic, substituent that can be a single ring or multiple rings (preferably from 1 to 3 rings), which are fused together or linked covalently. The term "heteroaryl" refers to aryl groups (or rings) that contain from one to four heteroatoms selected from N, O, and S, wherein the nitrogen and sulfur atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. A heteroaryl group can be attached to the remainder of the molecule through a heteroatom. Non-limiting examples of aryl and heteroaryl groups include phenyl, 1-naphthyl, 2-naphthyl, 4-biphenyl, 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2-imidazolyl, 4-imidazolyl, pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidyl, 4-pyrimidyl, 5-benzothiazolyl, purinyl, 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 5-isoquinolyl, 2-quinoxalinyl, 5-quinoxalinyl, 3-quinolyl, tetrazolyl, benzo[b]furanyl, benzo[b]thienyl, 2,3-dihydrobenzo[1,4]dioxin-6-yl, benzo[1,3]dioxol-5-yl and 6-quinolyl. Substituents for each of the above noted aryl and heteroaryl ring systems are selected from the group of acceptable substituents described below.

[0101] For brevity, the term "aryl" when used in combination with other terms (*e.g.*, aryloxy, arylthioxy, arylalkyl) includes both aryl and heteroaryl rings as defined above. Thus, the term "arylalkyl" is meant to include those radicals in which an aryl group is

attached to an alkyl group (*e.g.*, benzyl, phenethyl, pyridylmethyl and the like) including those alkyl groups in which a carbon atom (*e.g.*, a methylene group) has been replaced by, for example, an oxygen atom (*e.g.*, phenoxymethyl, 2-pyridyloxymethyl, 3-(1-naphthyloxy)propyl, and the like).

[0102] Each of the above terms (*e.g.*, "alkyl," "heteroalkyl," "aryl" and "heteroaryl") is meant to include both substituted and unsubstituted forms of the indicated radical. Preferred substituents for *each* type of radical are provided below.

[0103] Substituents for the alkyl and heteroalkyl radicals (including those groups often referred to as alkylene, alkenyl, heteroalkylene, heteroalkenyl, alkynyl, cycloalkyl, heterocycloalkyl, cycloalkenyl, and heterocycloalkenyl) are generically referred to as "alkyl group substituents," and they can be one or more of a variety of groups selected from, but not limited to: -OR', =O, =NR', =N-OR', -NR'R", -SR', -halogen, -SiR'R"R"', -OC(O)R', -C(O)R', -CO_2R', -CONR'R", -OC(O)NR'R", -NR"C(O)R', -NR'-C(O)NR"R"', -NR"$C(O)_2$R', -NR-C(NR'R"R"')=NR"", -NR-C(NR'R")=NR"', -S(O)R', -$S(O)_2$R', -$S(O)_2$NR'R", -$NRSO_2$R', -CN and –NO_2 in a number ranging from zero to (2m'+1), where m' is the total number of carbon atoms in such radical. R', R", R"' and R"" each preferably independently refer to hydrogen, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, *e.g.*, aryl substituted with 1-3 halogens, substituted or unsubstituted alkyl, alkoxy or thioalkoxy groups, or arylalkyl groups. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R", R"' and R"" groups when more than one of these groups is present. When R' and R" are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 5-, 6-, or 7-membered ring. For example, -NR'R" is meant to include, but not be limited to, 1-pyrrolidinyl and 4-morpholinyl. From the above discussion of substituents, one of skill in the art will understand that the term "alkyl" is meant to include groups including carbon atoms bound to groups other than hydrogen groups, such as haloalkyl (*e.g.*, -CF_3 and –CH_2CF_3) and acyl (*e.g.*, -C(O)CH_3, -C(O)CF_3, -C(O)CH_2OCH_3, and the like).

[0104] Similar to the substituents described for the alkyl radical, substituents for the aryl and heteroaryl groups are generically referred to as "aryl group substituents." The substituents are selected from, for example: halogen, -OR', =O, =NR', =N-OR', -NR'R", -SR', -halogen, -SiR'R"R"', -OC(O)R', -C(O)R', -CO_2R', -CONR'R", -OC(O)NR'R", -NR"C(O)R', -NR'-C(O)NR"R"', -NR"$C(O)_2$R', -NR-C(NR'R"R"')=NR"", -NR-C(NR'R")=NR"', -S(O)R', -$S(O)_2$R', -$S(O)_2$NR'R", -$NRSO_2$R', -CN and –NO_2, -R', -

N_3, -$CH(Ph)_2$, fluoro(C_1-C_4)alkoxy, and fluoro(C_1-C_4)alkyl, in a number ranging from zero to the total number of open valences on the aromatic ring system; and where R', R", R"' and R"" are preferably independently selected from hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl and substituted or unsubstituted heteroaryl. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R", R"' and R"" groups when more than one of these groups is present. In the schemes that follow, the symbol X represents "R" as described above.

[0105] Two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula –T-C(O)-$(CRR')_u$-U-, wherein T and U are independently –NR-, -O-, -CRR'- or a single bond, and u is an integer of from 0 to 3. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula –A-$(CH_2)_r$-B-, wherein A and B are independently –CRR'-, -O-, -NR-, -S-, -S(O)-, -$S(O)_2$-, -$S(O)_2$NR'- or a single bond, and r is an integer of from 1 to 4. One of the single bonds of the new ring so formed may optionally be replaced with a double bond. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula –$(CRR')_z$-X-$(CR"R"')_d$-, where z and d are independently integers of from 0 to 3, and X is -O-, -NR'-, -S-, -S(O)-, -$S(O)_2$-, or –$S(O)_2$NR'-. The substituents R, R', R" and R"' are preferably independently selected from hydrogen or substituted or unsubstituted (C_1-C_6)alkyl.

[0106] As used herein, the term "heteroatom" is meant to include oxygen (O), nitrogen (N), sulfur (S) and silicon (Si).

Introduction

[0107] FGF-9 is a fibroblast growth factor that is expressed as a secreted protein in the brain and the uterine endometrium. The 208 amino acid heparin binding protein is thought to be unglycosylated in its wild-type state. As an autocrine/paracrine growth factor, FGF-9 plays an important role in glial cell development and in the proliferation and activation of other cells expressing FGF receptors, such as those found in the motorneurons and prostate.

FGF-18 is another member of the family of the FGF family. It is involved in stimulating hepatic and intestinal proliferation and is an essential regulator of bone and cartilage differentiation. Like FGF-9, it too is thought to be unglycosylated in its wild-type state. This 207 amino acid protein is also involved in postnatal lung development through the

stimulation of myofibroblast proliferation and differentiation. Induced by calcineurin, FGF-18 has the ability to repress noggin expression and act as an effective neuroprotective agent.

FGF-20 is a novel fibroblast growth factor that is expressed as a secreted protein in the brain (*e.g.*, cerebellum and substantia nigra pars compacta) and expressed in *E. coli* as a monomer of apparent molecular weight of 23 kDa. This 211 amino acid heparin binding protein is thought to be un-glycosylated in its wild-type state. Its biological activities include neurogenesis, neuroprotection, CNS regeneration, anti-inflammatory effects (*e.g.*, bowel anti-inflammatory agent) and wound healing, making it a useful agent for treating diseases such as Parkinson's and Alzheimer's. FGF-20 can also be used as a prophylactic or mitigating agent against radiation toxicity to the GI and other parts of the body, *e.g.* arising from chemo- and radiation therapy, nuclear/radiological terrorism, radiation accidents, etc. In several studies, FGF-20 has also demonstrated its effectiveness in preventing and treating oral mucositis, a condition characterized by symptoms ranging from mild erythema to severe painful ulcerations.

[0108] FGF-21, another novel fibroblast growth factor, is expressed in liver, thymic and testicular tissue. The 209 amino acid protein is also thought to be unglycosylated in the wild-type state. In recent studies, FGF-21 was shown to regulate glucose uptake in human fat cells, suggesting its role as a metabolic regulator. Its effect on insulin activity and its regulation of lipidolysis make FGF-21 a useful treatment for type II diabetes and obesity. It has been implicated in various diseases characterized by complete or partial loss of cellular, tissue, or organ function as well as abnormalities in the function or number of cells and/or tissue. FGF-21 also has numerous other therapeutic applications, as will be described below.

[0109] One disease amenable to FGF-21 treatment is ischemic vascular disease. Treatment with the peptide may induce therapeutic angiogenesis or preserve function/survival of cells in patients suffering from diseases such as myocardial ischemia/infarction, peripheral vascular disease, renal artery disease, or stroke, etc.

[0110] Other diseases for which FGF-21 therapy can be useful include cardiomyopathies, which are characterized by loss of function or death of cardiac myocytes or supporting cells in the heart as occurs in, *e.g.* congestive heart failure, myocarditis, and musculoskeletal diseases, which are characterized by loss of function, inadequate function or death of skeletal muscle cells, bone cells or supporting cells, *e.g.* skeletal myopathies, bone disease, and

arthritis. In addition, congenital defects in, *e.g.* liver, heart, lung, brain, limbs, kidney, etc., arising from the loss of FGF-21 or its function are treatable with FGF-21.

[0111] FGF-21 polypeptides and polynucleotides can also facilitate the healing of wounds originating from trauma, disease, medical or surgical treatment, and aid in cell and tissue regeneration necessitated by the above circumstances. For example, FGF-21 can effect liver regeneration, operative wound healing, re-endothelialization of injured blood vessels, healing of traumatic wounds, healing of ulcers due to vascular, metabolic disease, etc., bone fractures, loss of cells due to inflammatory disease, etc.

[0112] To improve the effectiveness of recombinant FGF used for therapeutic purposes, the present invention provides conjugates of FGF peptides with a modifying group. Some of the peptides in these FGF peptide conjugates have the same amino acid or nucleotide sequence as the wild-type FGF, while others are mutants.

[0113] The modifying groups can be selected from polymeric modifying groups such as, *e.g.*, PEG (m-PEG), PPG (m-PPG), etc., therapeutic moieties, diagnostic moieties, targeting moieties and the like. Creation of a FGF peptide conjugate, *e.g.*, adding a water-soluble polymeric modifying group, can improve the stability and retention time of FGF in a patient's circulation, and/or reduce the antigenicity of FGF.

[0114] The peptide conjugates of the invention can be formed by the enzymatic attachment of a modified sugar to a glycosylated or unglycosylated peptide. An amino acid glycosylation site and/or a glycosyl group provides a locus for conjugating a modified sugar bearing a modifying group to the peptide, *e.g.*, by glycoconjugation.

[0115] The present invention also provides genetically engineered mutants of Fibroblast Growth Factor that contain N-linked or O-linked glycosylation sites not present in naturally occurring Fibroblast Growth Factor. While these FGF mutants substantially retain the biological activity of the wild-type hormone, the newly introduced glycosylation sites allow the recombinantly produced FGF mutants to be glycosylated in a large variety of patterns.

[0116] The methods of the invention also make it possible to assemble peptide conjugates and glycopeptide conjugates that have a substantially homogeneous derivatization pattern. The enzymes used in the invention are generally selective for a particular amino acid residue, combination of amino acid residues, particular glycosyl residues, or combination of glycosyl residues of the peptide. The methods are also practical for large-scale production of peptide

conjugates. Thus, the methods of the invention provide a practical means for large-scale preparation of peptide conjugates having preselected uniform derivatization patterns. The methods are particularly well suited for modification of therapeutic peptides, including but not limited to, glycopeptides that are incompletely glycosylated during production in cell culture cells (*e.g.*, mammalian cells, insect cells, plant cells, fungal cells, yeast cells, or prokaryotic cells) or transgenic plants or animals.

[0117] The FGF peptide conjugates can be included in pharmaceutical formulations comprising a FGF peptide conjugate as well as a pharmaceutically acceptable carrier.

[0118] The present invention also provides conjugates of FGF peptides with increased therapeutic half-life due to, for example, reduced clearance rate, or reduced rate of uptake by the immune or reticuloendothelial system (RES). Moreover, the methods of the invention provide a means for masking antigenic determinants on peptides, thus reducing or eliminating a host immune response against the peptide. Selective attachment of targeting agents can also be used to target a peptide to a particular tissue or cell surface receptor that is specific for the particular targeting agent.

The Mutants

[0119] The present invention provides mutants of FGF that include one or more O- or N-linked glycosylation sites that are not found in the wild type peptide. The mutants are substrates for enzymatic glycosylation at one or more sites that would not normally be glycosylated, or would be poorly glycosylated, in the wild type peptide. Thus, the mutants allow the position of a glycosyl residue or a glycosyl linking group to be engineered to obtain a peptide having selected desirable properties. In addition to the position and number of glycosyl residues or glycosyl linking groups, other properties that can be varied using the mutants and methods of the invention include pharmacokinetics, pharmacodynamics, resistance to proteolysis, immunogenicity, recognition by the reticuloendothelial system, tissue distribution and the like.

[0120] Accordingly, in one aspect, the present invention provides an isolated nucleic acid comprising a polynucleotide sequence encoding a mutant Fibroblast Growth Factor. The mutant Fibroblast Growth Factor comprises an N-linked or O-linked glycosylation site that does not exist in the corresponding wild-type Fibroblast Growth Factor. In some embodiments, the corresponding wild-type Fibroblast Growth Factor has the amino acid

sequence of SEQ ID NO:1. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, and 112-145. In some other embodiments, the wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:146. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 161-214, 220-320, and 323-360. In an exemplary embodiment, a peptide having Fibroblast Growth Factor activity has an amino acid sequence that is at least about 95% homologous to the amino acid sequences set forth herein. Perferably, the amino acid sequence is at least about 96%, 97%, 98% or 99% homologous to the amino acid sequences set forth herein.

[0121] In another aspect, the present invention provides an expression cassette or a cell that comprises a nucleic acid, *e.g.*, an isolated nucleic acid, including a polynucleotide sequence encoding a mutant Fibroblast Growth Factor. The mutant Fibroblast Growth Factor includes one or more N-linked or O-linked glycosylation site that does not exist in the corresponding wild-type Fibroblast Growth Factor.

[0122] In another aspect, the present invention provides a mutant Fibroblast Growth Factor, that includes an N-linked or O-linked glycosylation site that does not exist in the corresponding wild-type Fibroblast Growth Factor. In some embodiments, the corresponding wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:1. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, and 112-145. In some other embodiments, the wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:146. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 161-214, 220-320, and 323-360. In an exemplary embodiment, a peptide having Fibroblast Growth Factor activity has an amino acid sequence that is at least about 95% homologous to the amino acid sequences set forth herein. Perferably, the amino acid sequence is at least about 96%, 97%, 98% or 99% homologous to the amino acid sequences set forth herein.

[0123] In another aspect, the present invention provides a method for making a mutant Fibroblast Growth Factor that includes an N-linked or O-linked glycosylation site that does not exist in the corresponding wild-type Fibroblast Growth Factor. This method comprises the steps of recombinantly producing the mutant Fibroblast Growth Factor, and glycosylating

the mutant Fibroblast Growth Factor at the new glycosylation site. In some embodiments, the corresponding wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:1. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, and 112-145. In some other embodiments, the wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:146. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 161-214, 220-320, and 323-360. In an exemplary embodiment, a peptide having Fibroblast Growth Factor activity has an amino acid sequence that is at least about 95% homologous to the amino acid sequences set forth herein. Perferably, the amino acid sequence is at least about 96%, 97%, 98% or 99% homologous to the amino acid sequences set forth herein.

Acquisition of FGF Coding Sequences

General Recombinant Technology

[0124] This invention relies on routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook and Russell, *Molecular Cloning, A Laboratory Manual* (3rd ed. 2001); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and Ausubel *et al.*, eds., *Current Protocols in Molecular Biology* (1994).

[0125] For nucleic acids, sizes are given in either kilobases (kb) or base pairs (bp). These are estimates derived from agarose or acrylamide gel electrophoresis, from sequenced nucleic acids, or from published DNA sequences. For proteins, sizes are given in kilodaltons (kDa) or amino acid residue numbers. Proteins sizes are estimated from gel electrophoresis, from sequenced proteins, from derived amino acid sequences, or from published protein sequences.

[0126] Oligonucleotides that are not commercially available can be chemically synthesized, *e.g.*, according to the solid phase phosphoramidite triester method first described by Beaucage & Caruthers, *Tetrahedron Lett.* **22**: 1859-1862 (1981), using an automated synthesizer, as described in Van Devanter *et. al.*, *Nucleic Acids Res.* **12**: 6159-6168 (1984). Purification of oligonucleotides is performed using any art-recognized strategy, *e.g.*, native acrylamide gel electrophoresis or anion-exchange HPLC as described in Pearson & Reanier, *J. Chrom.* **255**: 137-149 (1983).

[0127] The sequence of the cloned wild-type Fibroblast Growth Factor genes, polynucleotide encoding mutant Fibroblast Growth Factors, and synthetic oligonucleotides

can be verified after cloning using, *e.g.*, the chain termination method for sequencing double-stranded templates of Wallace *et al.*, *Gene* **16**: 21-26 (1981).

Cloning and Subcloning of a Wild-Type FGF Coding Sequence

[0128] A number of polynucleotide sequences encoding a wild-type Fibroblast Growth Factor-20, *e.g.*, GenBank Accession No. NM_019851, NM_019113, have been determined and can be obtained from a commercial supplier.

[0129] The rapid progress in the studies of human genome has made possible a cloning approach where a human DNA sequence database can be searched for any gene segment that has a certain percentage of sequence homology to a known nucleotide sequence, such as one encoding a previously identified Fibroblast Growth Factor. Any DNA sequence so identified can be subsequently obtained by chemical synthesis and/or a polymerase chain reaction (PCR) technique such as overlap extension method. For a short sequence, completely *de novo* synthesis may be sufficient; whereas further isolation of full length coding sequence from a human cDNA or genomic library using a synthetic probe may be necessary to obtain a larger gene.

[0130] Alternatively, a nucleic acid sequence encoding a Fibroblast Growth Factor can be isolated from a human cDNA or genomic DNA library using standard cloning techniques such as polymerase chain reaction (PCR), where homology-based primers can often be derived from a known nucleic acid sequence encoding a Fibroblast Growth Factor. Most commonly used techniques for this purpose are described in standard texts, *e.g.*, Sambrook and Russell, *supra*.

[0131] cDNA libraries suitable for obtaining a coding sequence for a wild-type Fibroblast Growth Factor may be commercially available or can be constructed. The general methods of isolating mRNA, making cDNA by reverse transcription, ligating cDNA into a recombinant vector, transfecting into a recombinant host for propagation, screening, and cloning are well known (*see*, *e.g.*, Gubler and Hoffman, *Gene*, **25**: 263-269 (1983); Ausubel *et al.*, *supra*). Upon obtaining an amplified segment of nucleotide sequence by PCR, the segment can be further used as a probe to isolate the full length polynucleotide sequence encoding the wild-type Fibroblast Growth Factor from the cDNA library. A general description of appropriate procedures can be found in Sambrook and Russell, *supra*.

[0132] A similar procedure can be followed to obtain a full length sequence encoding a wild-type Fibroblast Growth Factor, *e.g.*, any one of the GenBank Accession Nos. mentioned

above, from a human genomic library. Human genomic libraries are commercially available or can be constructed according to various art-recognized methods. In general, to construct a genomic library, the DNA is first extracted from an tissue where a Fibroblast Growth Factor is likely found. The DNA is then either mechanically sheared or enzymatically digested to yield fragments of about 12-20 kb in length. The fragments are subsequently separated by gradient centrifugation from polynucleotide fragments of undesired sizes and are inserted in bacteriophage λ vectors. These vectors and phages are packaged *in vitro*. Recombinant phages are analyzed by plaque hybridization as described in Benton and Davis, *Science*, **196**: 180-182 (1977). Colony hybridization is carried out as described by Grunstein *et al.*, *Proc. Natl. Acad. Sci. USA*, **72**: 3961-3965 (1975).

[0133] Based on sequence homology, degenerate oligonucleotides can be designed as primer sets and PCR can be performed under suitable conditions (*see, e.g.*, White *et al.*, *PCR Protocols: Current Methods and Applications*, 1993; Griffin and Griffin, *PCR Technology*, CRC Press Inc. 1994) to amplify a segment of nucleotide sequence from a cDNA or genomic library. Using the amplified segment as a probe, the full length nucleic acid encoding a wild-type Fibroblast Growth Factor is obtained.

[0134] Upon acquiring a nucleic acid sequence encoding a wild-type Fibroblast Growth Factor, the coding sequence can be subcloned into a vector, for instance, an expression vector, so that a recombinant wild-type Fibroblast Growth Factor can be produced from the resulting construct. Further modifications to the wild-type Fibroblast Growth Factor coding sequence, *e.g.*, nucleotide substitutions, may be subsequently made to alter the characteristics of the molecule.

Introducing Mutations into an FGF Sequence

[0135] From an encoding polynucleotide sequence, the amino acid sequence of a wild-type Fibroblast Growth Factor, *e.g.*, SEQ ID NO:1, SEQ ID NO:146, can be determined. Subsequently, this amino acid sequence may be modified to alter the protein's glycosylation pattern, by introducing additional glycosylation site(s) at various locations in the amino acid sequence.

[0136] Several types of protein glycosylation sites are well known in the art. For instance, in eukaryotes, N-linked glycosylation occurs on the asparagine of the consensus sequence Asn-X_{aa}-Ser/Thr, in which X_{aa} is any amino acid except proline (Kornfeld et al., *Ann Rev Biochem* **54**:631-664 (1985); Kukuruzinska *et al.*, *Proc. Natl. Acad. Sci. USA* **84**:2145-2149

(1987); Herscovics *et al.*, *FASEB J* 7:540-550 (1993); and Orlean, *Saccharomyces* **Vol. 3** (1996)). O-linked glycosylation takes place at serine or threonine residues (Tanner *et al.*, *Biochim. Biophys. Acta.* **906**:81-91 (1987); and Hounsell *et al.*, *Glycoconj. J.* **13**:19-26 (1996)). Other glycosylation patterns are formed by linking glycosylphosphatidylinositol to the carboxyl-terminal carboxyl group of the protein (Takeda *et al.*, *Trends Biochem. Sci.* **20**:367-371 (1995); and Udenfriend *et al.*, *Ann. Rev. Biochem.* **64**:593-591 (1995). Based on this knowledge, suitable mutations can thus be introduced into a wild-type Fibroblast Growth Factor sequence to form new glycosylation sites.

[0137] Although direct modification of an amino acid residue within a Fibroblast Growth Factor polypeptide sequence may be suitable to introduce a new N-linked or O-linked glycosylation site, more frequently, introduction of a new glycosylation site is accomplished by mutating the polynucleotide sequence encoding a Fibroblast Growth Factor. This can be achieved by using any of known mutagenesis methods, some of which are discussed below. Exemplary modifications to Fibroblast Growth Factor include those illustrated in SEQ ID NO:9 or SEQ ID NO:87.

[0138] A variety of mutation-generating protocols are established and described in the art. *See, e.g.*, Zhang et al., *Proc. Natl. Acad. Sci. USA*, **94**: 4504-4509 (1997); and Stemmer, *Nature*, **370**: 389-391 (1994). The procedures can be used separately or in combination to produce variants of a set of nucleic acids, and hence variants of encoded polypeptides. Kits for mutagenesis, library construction, and other diversity-generating methods are commercially available.

[0139] Mutational methods of generating diversity include, for example, site-directed mutagenesis (Botstein and Shortle, *Science*, **229**: 1193-1201 (1985)), mutagenesis using uracil-containing templates (Kunkel, *Proc. Natl. Acad. Sci. USA*, **82**: 488-492 (1985)), oligonucleotide-directed mutagenesis (Zoller and Smith, *Nucl. Acids Res.*, **10**: 6487-6500 (1982)), phosphorothioate-modified DNA mutagenesis (Taylor *et al.*, *Nucl. Acids Res.*, **13**: 8749-8764 and 8765-8787 (1985)), and mutagenesis using gapped duplex DNA (Kramer *et al.*, *Nucl. Acids Res.*, **12**: 9441-9456 (1984)).

[0140] Other possible methods for generating mutations include point mismatch repair (Kramer *et al.*, *Cell*, **38**: 879-887 (1984)), mutagenesis using repair-deficient host strains (Carter *et al.*, *Nucl. Acids Res.*, **13**: 4431-4443 (1985)), deletion mutagenesis (Eghtedarzadeh and Henikoff, *Nucl. Acids Res.*, **14**: 5115 (1986)), restriction-selection and restriction-

purification (Wells *et al.*, *Phil. Trans. R. Soc. Lond. A*, **317**: 415-423 (1986)), mutagenesis by total gene synthesis (Nambiar *et al.*, *Science*, **223**: 1299-1301 (1984)), double-strand break repair (Mandecki, *Proc. Natl. Acad. Sci. USA*, **83**: 7177-7181 (1986)), mutagenesis by polynucleotide chain termination methods (U.S. Patent No. 5,965,408), and error-prone PCR (Leung *et al.*, *Biotechniques*, **1**: 11-15 (1989)).

Modification of Nucleic Acids for Preferred Codon Usage in a Host Organism

[0141] The polynucleotide sequence encoding a mutant Fibroblast Growth Factor can be further altered to coincide with the preferred codon usage of a particular host. For example, the preferred codon usage of one strain of bacterial cells can be used to derive a polynucleotide that encodes a mutant Fibroblast Growth Factor of the invention and includes the codons favored by this strain. The frequency of preferred codon usage exhibited by a host cell can be calculated by averaging frequency of preferred codon usage in a large number of genes expressed by the host cell (*e.g.*, calculation service is available from web site of the Kazusa DNA Research Institute, Japan). This analysis is preferably limited to genes that are highly expressed by the host cell. U.S. Patent No. 5,824,864, for example, provides the frequency of codon usage by highly expressed genes exhibited by dicotyledonous plants and monocotyledonous plants.

[0142] At the completion of modification, the mutant Fibroblast Growth Factor coding sequences are verified by sequencing and are then subcloned into an appropriate expression vector for recombinant production in the same manner as the wild-type Fibroblast Growth Factors.

Expression and Purification of the Mutant FGF

[0143] Following sequence verification, the mutant Fibroblast Growth Factor of the present invention can be produced using routine techniques in the field of recombinant genetics, relying on the polynucleotide sequences encoding the polypeptide disclosed herein.

Expression Systems

[0144] To obtain high level expression of a nucleic acid encoding a mutant Fibroblast Growth Factor of the present invention, one typically subclones a polynucleotide encoding the mutant Fibroblast Growth Factor into an expression vector that contains a strong promoter to direct transcription, a transcription/translation terminator and a ribosome binding site for translational initiation. Suitable bacterial promoters are well known in the art and described, *e.g.*, in Sambrook and Russell, *supra,* and Ausubel *et al.*, *supra*. Bacterial

expression systems for expressing the wild-type or mutant Fibroblast Growth Factor are available in, *e.g.*, *E. coli, Bacillus sp., Salmonella*, and *Caulobacter*. Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available. In one embodiment, the eukaryotic expression vector is an adenoviral vector, an adeno-associated vector, or a retroviral vector.

[0145] The promoter used to direct expression of a heterologous nucleic acid depends on the particular application. The promoter is optionally positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

[0146] In addition to the promoter, the expression vector typically includes a transcription unit or expression cassette that contains all the additional elements required for the expression of the mutant Fibroblast Growth Factor in host cells. A typical expression cassette thus contains a promoter operably linked to the nucleic acid sequence encoding the mutant Fibroblast Growth Factor and signals required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. The nucleic acid sequence encoding the Fibroblast Growth Factor is typically linked to a cleavable signal peptide sequence to promote secretion of the Fibroblast Growth Factor by the transformed cell. Such signal peptides include, among others, the signal peptides from tissue plasminogen activator, insulin, and neuron growth factor, and juvenile hormone esterase of *Heliothis virescens*. Additional elements of the cassette may include enhancers and, if genomic DNA is used as the structural gene, introns with functional splice donor and acceptor sites.

[0147] In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

[0148] The particular expression vector used to transport the genetic information into the cell is not particularly critical. Any of the conventional vectors used for expression in eukaryotic or prokaryotic cells may be used. Standard bacterial expression vectors include plasmids such as pBR322 based plasmids, pSKF, pET23D, and fusion expression systems

such as GST and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, *e.g.*, c-myc.

[0149] Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, *e.g.*, SV40 vectors, papilloma virus vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A^+, pMTO10/A^+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

[0150] Some expression systems have markers that provide gene amplification such as thymidine kinase, hygromycin B phosphotransferase, and dihydrofolate reductase. Alternatively, high yield expression systems not involving gene amplification are also suitable, such as a baculovirus vector in insect cells, with a polynucleotide sequence encoding the mutant Fibroblast Growth Factor under the direction of the polyhedrin promoter or other strong baculovirus promoters.

[0151] The elements that are typically included in expression vectors also include a replicon that functions in *E. coli*, a gene encoding antibiotic resistance to permit selection of bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the plasmid to allow insertion of eukaryotic sequences. The particular antibiotic resistance gene chosen is not critical, any of the many resistance genes known in the art are suitable. The prokaryotic sequences are optionally chosen such that they do not interfere with the replication of the DNA in eukaryotic cells, if necessary. Similar to antibiotic resistance selection markers, metabolic selection markers based on known metabolic pathways may also be used as a means for selecting transformed host cells.

[0152] When periplasmic expression of a recombinant protein (*e.g.*, a FGF mutant of the present invention) is desired, the expression vector further comprises a sequence encoding a secretion signal, such as the E. coli OppA (Periplasmic Oligopeptide Binding Protein) secretion signal or a modified version thereof, which is directly connected to 5' of the coding sequence of the protein to be expressed. This signal sequence directs the recombinant protein produced in cytoplasm through the cell membrane into the periplasmic space. The expression vector may further comprise a coding sequence for signal peptidase 1, which is capable of

enzymatically cleaving the signal sequence when the recombinant protein is entering the periplasmic space. More detailed description for periplasmic production of a recombinant protein can be found in, *e.g.*, Gray *et al.*, *Gene* **39**: 247-254 (1985), U.S. Patent Nos. 6,160,089 and 6,436,674.

[0153] As discussed above, a person skilled in the art will recognize that various conservative substitutions can be made to any wild-type or mutant Fibroblast Growth Factor or its coding sequence while still retaining the biological activity of the Fibroblast Growth Factor. Moreover, modifications of a polynucleotide coding sequence may also be made to accommodate preferred codon usage in a particular expression host without altering the resulting amino acid sequence.

Transfection Methods

[0154] Standard transfection methods are used to produce bacterial, mammalian, yeast, insect, or plant cell lines that express large quantities of the mutant Fibroblast Growth Factor, which are then purified using standard techniques (*see, e.g.*, Colley *et al.*, *J. Biol. Chem.* **264**: 17619-17622 (1989); *Guide to Protein Purification*, in *Methods in Enzymology*, vol. 182 (Deutscher, ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques (*see, e.g.*, Morrison, *J. Bact.* **132**: 349-351 (1977); Clark-Curtiss & Curtiss, *Methods in Enzymology* **101**: 347-362 (Wu *et al.*, eds, 1983).

[0155] Any of the well known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, liposomes, microinjection, plasma vectors, viral vectors and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA, or other foreign genetic material into a host cell (*see, e.g.*, Sambrook and Russell, *supra*). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing the mutant Fibroblast Growth Factor.

Detection of Expression of Mutant FGF in Host Cells

[0156] After the expression vector is introduced into appropriate host cells, the transfected cells are cultured under conditions favoring expression of the mutant Fibroblast Growth Factor. The cells are then screened for the expression of the recombinant polypeptide, which is subsequently recovered from the culture using standard techniques (*see*, *e.g.*, Scopes,

Protein Purification: Principles and Practice (1982); U.S. Patent No. 4,673,641; Ausubel *et al., supra*; and Sambrook and Russell, *supra*).

[0157] Several general methods for screening gene expression are well known among those skilled in the art. First, gene expression can be detected at the nucleic acid level. A variety of methods of specific DNA and RNA measurement using nucleic acid hybridization techniques are commonly used (*e.g.*, Sambrook and Russell, *supra*). Some methods involve an electrophoretic separation (*e.g.*, Southern blot for detecting DNA and Northern blot for detecting RNA), but detection of DNA or RNA can be carried out without electrophoresis as well (such as by dot blot). The presence of nucleic acid encoding a mutant Fibroblast Growth Factor in transfected cells can also be detected by PCR or RT-PCR using sequence-specific primers.

[0158] Second, gene expression can be detected at the polypeptide level. Various immunological assays are routinely used by those skilled in the art to measure the level of a gene product, particularly using polyclonal or monoclonal antibodies that react specifically with a mutant Fibroblast Growth Factor of the present invention, such as a polypeptide having the amino acid sequence of SEQ ID NO:3, 4, or 5, (*e.g.*, Harlow and Lane, *Antibodies, A Laboratory Manual*, Chapter 14, Cold Spring Harbor, 1988; Kohler and Milstein, *Nature*, **256**: 495-497 (1975)). Such techniques require antibody preparation by selecting antibodies with high specificity against the mutant Fibroblast Growth Factor or an antigenic portion thereof. The methods of raising polyclonal and monoclonal antibodies are well established and their descriptions can be found in the literature, *see, e.g.*, Harlow and Lane, *supra*; Kohler and Milstein, *Eur. J. Immunol.*, **6**: 511-519 (1976). More detailed descriptions of preparing antibody against the mutant Fibroblast Growth Factor of the present invention and conducting immunological assays detecting the mutant Fibroblast Growth Factor are provided in a later section.

Purification of Recombinantly Produced Mutant FGF

[0159] Once the expression of a recombinant mutant Fibroblast Growth Factor in transfected host cells is confirmed, the host cells are then cultured in an appropriate scale for the purpose of purifying the recombinant polypeptide.

Purification of Recombinantly Produced Mutant FGF from Bacteria

[0160] When the mutant Fibroblast Growth Factors of the present invention are produced recombinantly by transformed bacteria in large amounts, typically after promoter induction,

although expression can be constitutive, the proteins may form insoluble aggregates. There are several protocols that are suitable for purification of protein inclusion bodies. For example, purification of aggregate proteins (hereinafter referred to as inclusion bodies) typically involves the extraction, separation and/or purification of inclusion bodies by disruption of bacterial cells, *e.g.*, by incubation in a buffer of about 100-150 μg/ml lysozyme and 0.1% Nonidet P40, a non-ionic detergent. The cell suspension can be ground using a Polytron grinder (Brinkman Instruments, Westbury, NY). Alternatively, the cells can be sonicated on ice. Alternate methods of lysing bacteria are described in Ausubel *et al.* and Sambrook and Russell, both *supra*, and will be apparent to those of skill in the art.

[0161] The cell suspension is generally centrifuged and the pellet containing the inclusion bodies resuspended in buffer which does not dissolve but washes the inclusion bodies, *e.g.*, 20 mM Tris-HCl (pH 7.2), 1 mM EDTA, 150 mM NaCl and 2% Triton-X 100, a non-ionic detergent. It may be necessary to repeat the wash step to remove as much cellular debris as possible. The remaining pellet of inclusion bodies may be resuspended in an appropriate buffer (*e.g.*, 20 mM sodium phosphate, pH 6.8, 150 mM NaCl). Other appropriate buffers will be apparent to those of skill in the art.

[0162] Following the washing step, the inclusion bodies are solubilized by the addition of a solvent that is both a strong hydrogen acceptor and a strong hydrogen donor (or a combination of solvents each having one of these properties). The proteins that formed the inclusion bodies may then be renatured by dilution or dialysis with a compatible buffer. Suitable solvents include, but are not limited to, urea (from about 4 M to about 8 M), formamide (at least about 80%, volume/volume basis), and guanidine hydrochloride (from about 4 M to about 8 M). Some solvents that are capable of solubilizing aggregate-forming proteins, such as SDS (sodium dodecyl sulfate) and 70% formic acid, may be inappropriate for use in this procedure due to the possibility of irreversible denaturation of the proteins, accompanied by a lack of immunogenicity and/or activity. Although guanidine hydrochloride and similar agents are denaturants, this denaturation is not irreversible and renaturation may occur upon removal (by dialysis, for example) or dilution of the denaturant, allowing re-formation of the immunologically and/or biologically active protein of interest. After solubilization, the protein can be separated from other bacterial proteins by standard separation techniques. For further description of purifying recombinant Fibroblast Growth Factor from bacterial inclusion body, *see, e.g.*, Patra *et al.*, *Protein Expression and Purification* **18**: 182-190 (2000).

[0163] Alternatively, it is possible to purify recombinant polypeptides, *e.g.*, a mutant Fibroblast Growth Factor, from bacterial periplasm. Where the recombinant protein is exported into the periplasm of the bacteria, the periplasmic fraction of the bacteria can be isolated by cold osmotic shock in addition to other methods known to those of skill in the art (*see e.g.*, Ausubel *et al.*, *supra*). To isolate recombinant proteins from the periplasm, the bacterial cells are centrifuged to form a pellet. The pellet is resuspended in a buffer containing 20% sucrose. To lyse the cells, the bacteria are centrifuged and the pellet is resuspended in ice-cold 5 mM $MgSO_4$ and kept in an ice bath for approximately 10 minutes. The cell suspension is centrifuged and the supernatant decanted and saved. The recombinant proteins present in the supernatant can be separated from the host proteins by standard separation techniques well known to those of skill in the art.

Standard Protein Separation Techniques for Purification

[0164] When a recombinant polypeptide, *e.g.*, the mutant Fibroblast Growth Factor of the present invention, is expressed in host cells in a soluble form, its purification can follow the standard protein purification procedure described below.

Solubility Fractionation

[0165] Often as an initial step, and if the protein mixture is complex, an initial salt fractionation can separate many of the unwanted host cell proteins (or proteins derived from the cell culture media) from the recombinant protein of interest, *e.g.*, a mutant Fibroblast Growth Factor of the present invention. The preferred salt is ammonium sulfate. Ammonium sulfate precipitates proteins by effectively reducing the amount of water in the protein mixture. Proteins then precipitate on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at lower ammonium sulfate concentrations. A typical protocol is to add saturated ammonium sulfate to a protein solution so that the resultant ammonium sulfate concentration is between 20-30%. This will precipitate the most hydrophobic proteins. The precipitate is discarded (unless the protein of interest is hydrophobic) and ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, through either dialysis or diafiltration. Other methods that rely on solubility of proteins, such as cold ethanol precipitation, are well known to those of skill in the art and can be used to fractionate complex protein mixtures.

Size Differential Filtration

[0166] Based on a calculated molecular weight, a protein of greater and lesser size can be isolated using ultrafiltration through membranes of different pore sizes (for example, Amicon or Millipore membranes). As a first step, the protein mixture is ultrafiltered through a membrane with a pore size that has a lower molecular weight cut-off than the molecular weight of a protein of interest, *e.g.*, a mutant Fibroblast Growth Factor. The retentate of the ultrafiltration is then ultrafiltered against a membrane with a molecular cut off greater than the molecular weight of the protein of interest. The recombinant protein will pass through the membrane into the filtrate. The filtrate can then be chromatographed as described below.

Column Chromatography

[0167] The proteins of interest (such as the mutant Fibroblast Growth Factor of the present invention) can also be separated from other proteins on the basis of their size, net surface charge, hydrophobicity, or affinity for ligands. In addition, antibodies raised against Fibroblast Growth Factor can be conjugated to column matrices and the Fibroblast Growth Factor immunopurified. All of these methods are well known in the art.

[0168] It will be apparent to one of skill that chromatographic techniques can be performed at any scale and using equipment from many different manufacturers (*e.g.*, Pharmacia Biotech).

Immunoassays for Detection of Mutant FGF Expression

[0169] To confirm the production of a recombinant mutant Fibroblast Growth Factor, immunological assays may be useful to detect in a sample the expression of the polypeptide. Immunological assays are also useful for quantifying the expression level of the recombinant hormone. Antibodies against a mutant Fibroblast Growth Factor are necessary for carrying out these immunological assays.

Production of Antibodies against Mutant FGF

[0170] Methods for producing polyclonal and monoclonal antibodies that react specifically with an immunogen of interest are known to those of skill in the art (*see, e.g.,* Coligan, *Current Protocols in Immunology* Wiley/Greene, NY, 1991; Harlow and Lane, *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY, 1989; Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY, 1986; and Kohler and Milstein *Nature* **256**: 495-497, 1975). Such

techniques include antibody preparation by selection of antibodies from libraries of recombinant antibodies in phage or similar vectors (*see*, Huse et al., *Science* **246**: 1275-1281, 1989; and Ward et al., *Nature* **341**: 544-546, 1989).

[0171] In order to produce antisera containing antibodies with desired specificity, the polypeptide of interest (*e.g.*, a mutant Fibroblast Growth Factor of the present invention) or an antigenic fragment thereof can be used to immunize suitable animals, *e.g.*, mice, rabbits, or primates. A standard adjuvant, such as Freund's adjuvant, can be used in accordance with a standard immunization protocol. Alternatively, a synthetic antigenic peptide derived from that particular polypeptide can be conjugated to a carrier protein and subsequently used as an immunogen.

[0172] The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the antigen of interest. When appropriately high titers of antibody to the antigen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich antibodies specifically reactive to the antigen and purification of the antibodies can be performed subsequently, *see*, Harlow and Lane, *supra*, and the general descriptions of protein purification provided above.

[0173] Monoclonal antibodies are obtained using various techniques familiar to those of skill in the art. Typically, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (*see*, Kohler and Milstein, *Eur. J. Immunol.* **6**:511-519, 1976). Alternative methods of immortalization include, *e.g.*, transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and the yield of the monoclonal antibodies produced by such cells may be enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host.

[0174] Additionally, monoclonal antibodies may also be recombinantly produced upon identification of nucleic acid sequences encoding an antibody with desired specificity or a binding fragment of such antibody by screening a human B cell cDNA library according to the general protocol outlined by Huse *et al.*, *supra*. The general principles and methods of recombinant polypeptide production discussed above are applicable for antibody production by recombinant methods.

[0175] When desired, antibodies capable of specifically recognizing a mutant Fibroblast Growth Factor of the present invention can be tested for their cross-reactivity against the wild-type Fibroblast Growth Factor and thus distinguished from the antibodies against the wild-type protein. For instance, antisera obtained from an animal immunized with a mutant Fibroblast Growth Factor can be run through a column on which a wild-type Fibroblast Growth Factor is immobilized. The portion of the antisera that passes through the column recognizes only the mutant Fibroblast Growth Factor and not the wild-type Fibroblast Growth Factor. Similarly, monoclonal antibodies against a mutant Fibroblast Growth Factor can also be screened for their exclusivity in recognizing only the mutant but not the wild-type Fibroblast Growth Factor.

[0176] Polyclonal or monoclonal antibodies that specifically recognize only the mutant Fibroblast Growth Factor of the present invention but not the wild-type Fibroblast Growth Factor are useful for isolating the mutant protein from the wild-type protein, for example, by incubating a sample with a mutant Fibroblast Growth Factor-specific polyclonal or monoclonal antibody immobilized on a solid support.

Immunoassays for Detecting Mutant FGF Expression

[0177] Once antibodies specific for a mutant Fibroblast Growth Factor of the present invention are available, the amount of the polypeptide in a sample, *e.g.*, a cell lysate, can be measured by a variety of immunoassay methods providing qualitative and quantitative results to a skilled artisan. For a review of immunological and immunoassay procedures in general *see, e.g.,* Stites, *supra;* U.S. Patent Nos. 4,366,241; 4,376,110; 4,517,288; and 4,837,168.

Glycosylation and Glycoconjugation of the Mutant FGF

Glycosylation and Glycoconjugation by Enzymatic Methods

[0178] Post-expression *in vitro* modification of peptides is an attractive strategy to remedy the deficiencies of methods that rely on controlling glycosylation by engineering expression systems; including both modification of glycan structures or introduction of glycans at novel sites. A comprehensive arsenal of enzymes that transfer saccharide donor moieties is becoming available, making *in vitro* enzymatic synthesis of glycoconjugates with custom designed glycosylation patterns and glycosyl structures possible. *See*, for example, U.S. Patent Nos. 5,876,980; 6,030,815; 5,728,554; 5,922,577; and published patent applications WO 98/31826; WO 01/88117; WO 03/031464; WO 03/046150; WO 03/045980; WO 03/093448; WO 04/009838; US2002/142370; US2003/040037; US2003/180835;

US2004/063911; US2003/207406; and US2003/124645, each of which is incorporated herein by reference.

[0179] The invention provides methods for preparing conjugates of glycosylated and unglycosylated mutant Fibroblast Growth Factors, which have glycosylation sites that do not exist in the corresponding wild-type FGF. Such conjugation may take place directly on the appropriate sugar units of a glycosylated mutant FGF, or following the removal (*i.e.*, "trimming back") of any undesired sugar units. The conjugates are formed between peptides and diverse species such as water-soluble polymers, therapeutic moieties, diagnostic moieties, targeting moieties and the like. Also provided are conjugates that include two or more peptides linked together through a linker arm, *i.e.*, multifunctional conjugates. The multi-functional conjugates of the invention can include two or more copies of the same peptide or a collection of diverse peptides with different structures, and/or properties.

[0180] The conjugates of the invention are formed by the enzymatic attachment of a modified sugar to the glycosylated or unglycosylated peptide. The modified sugar, when interposed between the peptide and the modifying group on the sugar becomes what is referred to herein as "an glycosyl linking group." Using the exquisite selectivity of enzymes, such as glycosyltransferases, the present method provides peptides that bear a desired group at one or more specific locations. Thus, according to the present invention, a modified sugar is attached directly to a selected locus on the peptide chain or, alternatively, the modified sugar is appended onto a carbohydrate moiety of a glycopeptide. Peptides in which modified sugars are bound to both a glycopeptide carbohydrate and directly to an amino acid residue of the peptide backbone are also within the scope of the present invention.

[0181] In contrast to known chemical and enzymatic peptide elaboration strategies, the methods of the invention, make it possible to assemble peptides and glycopeptides that have a substantially homogeneous derivatization pattern; the enzymes used in the invention are generally selective for a particular amino acid residue or combination of amino acid residues of the peptide. The methods are also practical for large-scale production of modified peptides and glycopeptides. Thus, the methods of the invention provide a practical means for large-scale preparation of glycopeptides having preselected uniform derivatization patterns. The methods are particularly well suited for modification of therapeutic peptides, including but not limited to, glycopeptides that are incompletely glycosylated during production in cell

culture cells (*e.g.*, mammalian cells, insect cells, plant cells, fungal cells, yeast cells, or prokaryotic cells) or transgenic plants or animals.

[0182] The methods of the invention also provide conjugates of glycosylated and unglycosylated peptides with increased therapeutic half-life due to, for example, reduced clearance rate, or reduced rate of uptake by the immune or reticuloendothelial system (RES). Moreover, the methods of the invention provide a means for masking antigenic determinants on peptides, thus reducing or eliminating a host immune response against the peptide. Selective attachment of targeting agents can also be used to target a peptide to a particular tissue or cell surface receptor that is specific for the particular targeting agent.

Peptide Conjugates

[0183] In another aspect, the present invention provides a conjugate between a modified sugar and a FGF peptide. The FGF peptide in these cases may have the same sequence as a wild-type peptide, or it may be a mutant peptide. A peptide conjugate can have one of several forms. In an exemplary embodiment, a peptide conjugate can comprise a FGF peptide and a modifying group linked to an amino acid of the peptide through a glycosyl linking group.

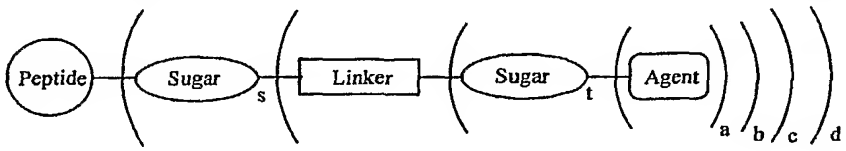
[0184] In another exemplary embodiment, a Fibroblast Growth Factor (FGF) peptide conjugate can comprise a FGF peptide and a glycosyl group attached to an amino acid residue of the FGF peptide. In another exemplary embodiment, the FGF peptide is a member selected from FGF-1, FGF-2, FGF-9, FGF-18, FGF-20 and FGF-21. In another exemplary embodiment, the FGF peptide comprises at least one amino acid sequence which is a member selected from SEQ ID NOs: 1, 9-14, 18-45, 48-65, 69-109, 112-145 and 146.

[0185] In an exemplary embodiment, the glycosyl group is an intact glycosyl linking group. In another exemplary embodiment, the glycosyl group further comprises a modifying group. In another exemplary embodiment, the modifying group is a non-glycosidic modifying group. In another exemplary embodiment, the modifying group does not include a naturally occurring saccharide moiety.

[0186] In another exemplary embodiment, the peptide conjugate can comprise a FGF peptide and a glycosyl linking group which is bound to both a glycopeptide carbohydrate and directly to an amino acid residue of the peptide backbone. In yet another exemplary embodiment, a peptide conjugate can comprise a FGF peptide and a modifying group linked

directly to an amino acid residue of the peptide. In this embodiment, the peptide conjugate may not comprise a glycosyl group. In any of these embodiments, the FGF peptide may or not be glycosylated. The present invention also encompasses a method for the modification of the glycan structure on FGF, providing a conjugate between FGF and a modifying group.

[0187] The conjugates of the invention will typically correspond to the general structure:



in which the symbols a, b, c, d and s represent a positive, non-zero integer; and t is either 0 or a positive integer. The "agent" is a therapeutic agent, a bioactive agent, a detectable label, water-soluble moiety (*e.g.*, PEG, m-PEG, PPG, and m-PPG) or the like. The "agent" can be a peptide, *e.g.*, enzyme, antibody, antigen, etc. The linker can be any of a wide array of linking groups, *infra*. Alternatively, the linker may be a single bond or a "zero order linker."

[0188] In the discussion that follows, the invention is illustrated by reference to the use of selected FGF peptides, such as FGF-20 and FGF-21. Those of skill in the art will recognize that the focus of the discussion is for clarity of illustration and that any FGF peptide, either wild-type or mutant, can be used to form these conjugates.

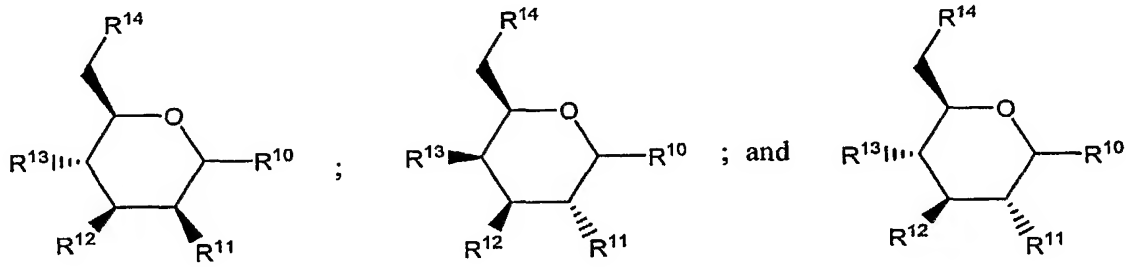
Modified Sugar

[0189] In an exemplary embodiment, the peptides of the invention are reacted with a modified sugar, thus forming a peptide conjugate. A modified sugar comprises a "sugar donor moiety" as well as a "sugar transfer moiety". The sugar donor moiety is any portion of the modified sugar that will be attached to the peptide, either through a glycosyl moiety or amino acid moiety, as a conjugate of the invention. The sugar donor moiety includes those atoms that are chemically altered during their conversion from the modified sugar to the glycosyl linking group of the peptide conjugate. The sugar transfer moiety is any portion of the modified sugar that will be not be attached to the peptide as a conjugate of the invention. For example, a modified sugar of the invention is the PEGylated sugar nucleotide, CMP-SA-PEG. For CMP-SA-PEG, the sugar donor moiety, or PEG-sialyl donor moiety, comprises PEG-sialic acid while the sugar transfer moiety, or sialyl transfer moiety, comprises CMP.

[0190] In modified sugars of use in the invention, the saccharyl moiety is preferably a saccharide, a deoxy-saccharide, an amino-saccharide, or an N-acyl saccharide. The term "saccharide" and its equivalents, "saccharyl," "sugar," and "glycosyl" refer to monomers,

dimers, oligomers and polymers. The sugar moiety is also functionalized with a modifying group. The modifying group is conjugated to the saccharyl moiety, typically, through conjugation with an amine, sulfhydryl or hydroxyl, *e.g.*, primary hydroxyl, moiety on the sugar. In an exemplary embodiment, the modifying group is attached through an amine moiety on the sugar, *e.g.*, through an amide, a urethane or a urea that is formed through the reaction of the amine with a reactive derivative of the modifying group.

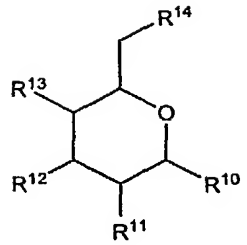
[0191] Any saccharyl moiety can be utilized as the sugar donor moiety of the modified sugar. The saccharyl moiety can be a known sugar, such as mannose, galactose or glucose, or a species having the stereochemistry of a known sugar. The general formulae of these modified sugars are:



Other saccharyl moieties that are useful in forming the compositions of the invention include, but are not limited to fucose and sialic acid, as well as amino sugars such as glucosamine, galactosamine, mannosamine, the 5-amine analogue of sialic acid and the like. The saccharyl moiety can be a structure found in nature or it can be modified to provide a site for conjugating the modifying group. For example, in one embodiment, the modified sugar provides a sialic acid derivative in which the 9-hydroxy moiety is replaced with an amine. The amine is readily derivatized with an activated analogue of a selected modifying group.

[0192] Examples of modified sugars of use in the invention are described in PCT Patent Application No. PCT/US05/002522, which is herein incorporated by reference.

[0193] In a further exemplary embodiment, the invention utilizes modified sugars in which the 6-hydroxyl position is converted to the corresponding amine moiety, which bears a linker-modifying group cassette such as those set forth above. Exemplary glycosyl groups that can be used as the core of these modified sugars include Gal, GalNAc, Glc, GlcNAc, Fuc, Xyl, Man, and the like. A representative modified sugar according to this embodiment has the formula:



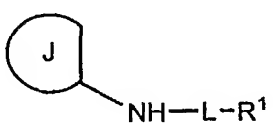
in which R^{11}-R^{14} are members independently selected from H, OH, $C(O)CH_3$, NH, and NH $C(O)CH_3$. R^{10} is a link to another glycosyl residue (-O-glycosyl) or to an amino acid of the Factor VII and/or Factor VIIa peptide (-NH-(Factor VII and/or Factor VIIa)). R^{14} is OR^1, NHR^1 or NH-L-R^1. R^1 and NH-L-R^1 are as described above.

Glycosyl Linking Groups

[0194] In an exemplary embodiment, the invention provides a peptide conjugate formed between a modified sugar of the invention and a FGF peptide. In this embodiment, the sugar donor moiety (such as the saccharyl moiety and the modifying group) of the modified sugar becomes a "glycosyl linking group". The "glycosyl linking group" can alternatively refer to the glycosyl moiety which is interposed between the peptide and the modifying group.

[0195] Due to the versatility of the methods available for adding and/or modifying glycosyl residues on a peptide, the glycosyl linking groups can have substantially any structure. In the discussion that follows, the invention is illustrated by reference to the use of selected derivatives of furanose and pyranose. Those of skill in the art will recognize that the focus of the discussion is for clarity of illustration and that the structures and compositions set forth are generally applicable across the genus of glycosyl linking groups and modified sugars. The glycosyl linking group can comprise virtually any mono- or oligo-saccharide. The glycosyl linking groups can be attached to an amino acid either through the side chain or through the peptide backbone. Alternatively the glycosyl linking groups can be attached to the peptide through a saccharyl moiety. This saccharyl moiety can be a portion of an O-linked or N-linked glycan structure on the peptide.

[0196] In an exemplary embodiment, the invention utilizes a glycosyl linking group that has the formula:



in which J is a glycosyl moiety, L is a bond or a linker and R^1 is a modifying group, *e.g.*, a polymeric modifying group. Exemplary bonds are those that are formed between an NH_2

moiety on the glycosyl moiety and a group of complementary reactivity on the modifying group. For example, when R^1 includes a carboxylic acid moiety, this moiety may be activated and coupled with the NH_2 moiety on the glycosyl residue affording a bond having the structure $NHC(O)R^1$. J is preferably a glycosyl moiety that is "intact", not having been degraded by exposure to conditions that cleave the pyranose or furanose structure, *e.g.* oxidative conditions, *e.g.*, sodium periodate.

[0197] Exemplary linkers include alkyl and heteroalkyl moieties. The linkers include linking groups, for example acyl-based linking groups, *e.g.*, -C(O)NH-, -OC(O)NH-, and the like. The linking groups are bonds formed between components of the species of the invention, *e.g.*, between the glycosyl moiety and the linker (L), or between the linker and the modifying group (R^1). Other exemplary linking groups are ethers, thioethers and amines. For example, in one embodiment, the linker is an amino acid residue, such as a glycine residue. The carboxylic acid moiety of the glycine is converted to the corresponding amide by reaction with an amine on the glycosyl residue, and the amine of the glycine is converted to the corresponding amide or urethane by reaction with an activated carboxylic acid or carbonate of the modifying group.

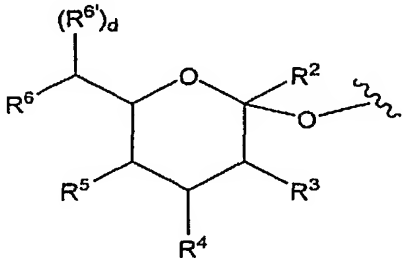
[0198] An exemplary species of NH-L-R^1 has the formula: $-NH\{C(O)(CH_2)_aNH\}_s\{C(O)(CH_2)_b(OCH_2CH_2)_cO(CH_2)_dNH\}_tR^1$, in which the indices s and t are independently 0 or 1. The indices a, b and d are independently integers from 0 to 20, and c is an integer from 1 to 2500. Other similar linkers are based on species in which an -NH moiety is replaced by another group, for example, -S, -O or $-CH_2$. As those of skill will appreciate one or more of the bracketed moieties corresponding to indices s and t can be replaced with a substituted or unsubstituted alkyl or heteroalkyl moiety.

[0199] More particularly, the invention utilizes compounds in which NH-L-R^1 is: $NHC(O)(CH_2)_aNHC(O)(CH_2)_b(OCH_2CH_2)_cO(CH_2)_dNHR^1$, $NHC(O)(CH_2)_b(OCH_2CH_2)_cO(CH_2)_dNHR^1$, $NHC(O)O(CH_2)_b(OCH_2CH_2)_cO(CH_2)_dNHR^1$, $NH(CH_2)_aNHC(O)(CH_2)_b(OCH_2CH_2)_cO(CH_2)_dNHR^1$, $NHC(O)(CH_2)_aNHR^1$, $NH(CH_2)_aNHR^1$, and NHR^1. In these formulae, the indices a, b and d are independently selected from the integers from 0 to 20, preferably from 1 to 5. The index c is an integer from 1 to about 2500.

[0200] In an exemplary embodiment, c is selected such that the PEG moiety is approximately 1 kD, 5 kD, 10, kD, 15 kD, 20 kD, 25 kD, 30 kD, 35 kD, 40 kD, 45 kD, 50 kD, 55 kD, 60 kD or 65 kD.

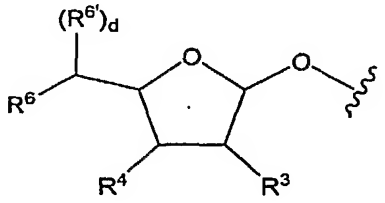
[0201] For the purposes of convenience, the glycosyl linking groups in the remainder of this section will be based on a sialyl moiety. However, one of skill in the art will recognize that another glycosyl moiety, such as mannosyl, galactosyl, glucosyl, or fucosyl, could be used in place of the sialyl moiety.

[0202] In an exemplary embodiment, the glycosyl linking group is an intact glycosyl linking group, in which the glycosyl moiety or moieties forming the linking group are not degraded by chemical (*e.g.*, sodium metaperiodate) or enzymatic (*e.g.*, oxidase) processes. Selected conjugates of the invention include a modifying group that is attached to the amine moiety of an amino-saccharide, *e.g.*, mannosamine, glucosamine, galactosamine, sialic acid etc. In an exemplary embodiment, the invention provides a peptide conjugate comprising an intact glycosyl linking group having a formula that is selected from:



I

; and

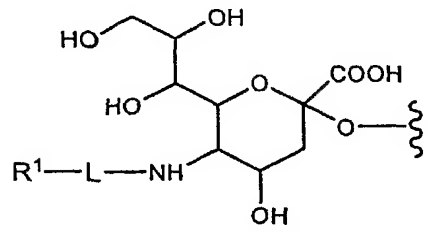


II

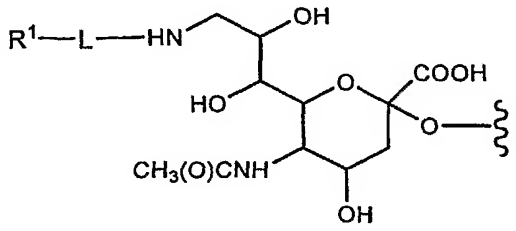
In Formulae I R^2 is H, CH_2OR^7, $COOR^7$ or OR^7, in which R^7 represents H, substituted or unsubstituted alkyl or substituted or unsubstituted heteroalkyl. When $COOR^7$ is a carboxylic acid or carboxylate, both forms are represented by the designation of the single structure COO^- or COOH. In Formulae I and II, the symbols R^3, R^4, R^5, R^6 and $R^{6'}$ independently represent H, substituted or unsubstituted alkyl, OR^8, $NHC(O)R^9$. The index d is 0 or 1. R^8 and R^9 are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, sialic acid or polysialic acid. At least one of R^3, R^4, R^5, R^6 or $R^{6'}$ includes a modifying group. This modifying group can be a polymeric modifying moiety *e.g.*, PEG, linked through a bond or a linking group. In an exemplary embodiment, R^6 and $R^{6'}$, together with the carbon to which they are attached are components of the pyruvyl side chain of sialic acid. In a further exemplary embodiment, the pyruvyl side chain is functionalized with the polymeric modifying group. In another exemplary embodiment, R^6

and $R^{6'}$, together with the carbon to which they are attached are components of the side chain of sialic acid and the polymeric modifying group is a component of R^5.

[0203] Exemplary modifying group-intact glycosyl linking group cassettes according to this motif are based on a sialic acid structure, such as those having the formulae:

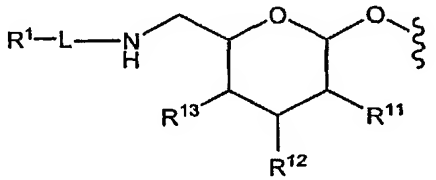


; and



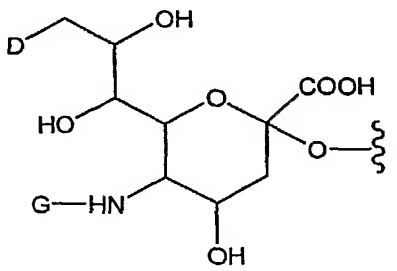
[0204] In the formulae above, R^1 and L are as described above. Further detail about the structure of exemplary R^1 groups is provided below.

[0205] In still a further exemplary embodiment, the conjugate is formed between a peptide and a modified sugar in which the modifying group is attached through a linker at the 6-carbon position of the modified sugar. Thus, illustrative glycosyl linking groups according to this embodiment have the formula:



in which the radicals are as discussed above. Glycosyl linking groups include, without limitation, glucose, glucosamine, N-acetyl-glucosamine, galactose, galactosamine, N-acetyl-galactosamine, mannose, mannosamine, N-acetyl-mannosamine, and the like.

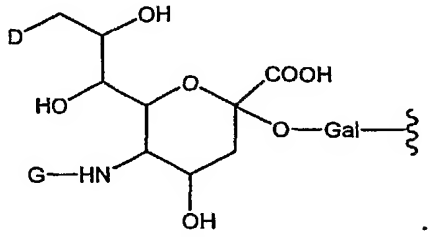
[0206] In one embodiment, the present invention provides a peptide conjugate comprising the following glycosyl linking group:



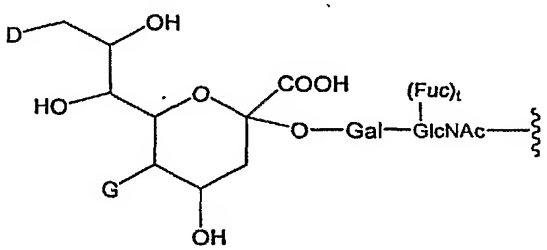
wherein D is a member selected from -OH and R^1-L-HN-; G is a member selected from H and R^1-L- and -C(O)(C_1-C_6)alkyl; R^1 is a moiety comprising a straight-chain or branched

poly(ethylene glycol) residue; and L is a linker, *e.g.*, a bond ("zero order"), substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl. In exemplary embodiments, when D is OH, G is R^1-L-, and when G is –C(O)(C_1-C_6)alkyl, D is R^1-L-NH-.

[0207] The invention provides a peptide conjugate that includes a glycosyl linking group having the formula:

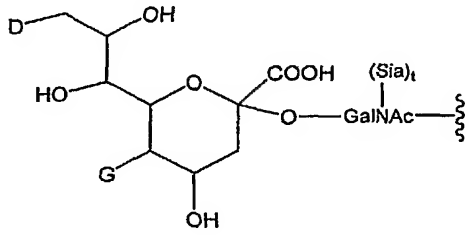


[0208] In other embodiments, the glycosyl linking group has the formula:



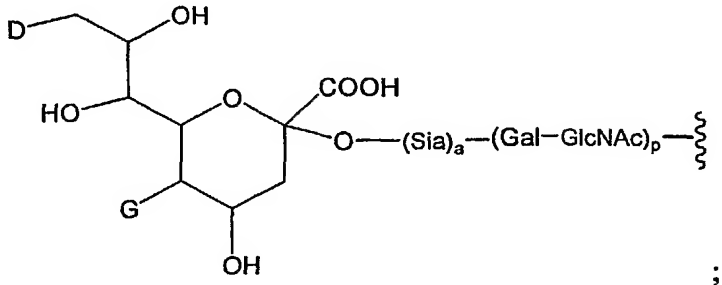
in which the index t is 0 or 1.

[0209] In a still further exemplary embodiment, the glycosyl linking group has the formula:



in which the index t is 0 or 1.

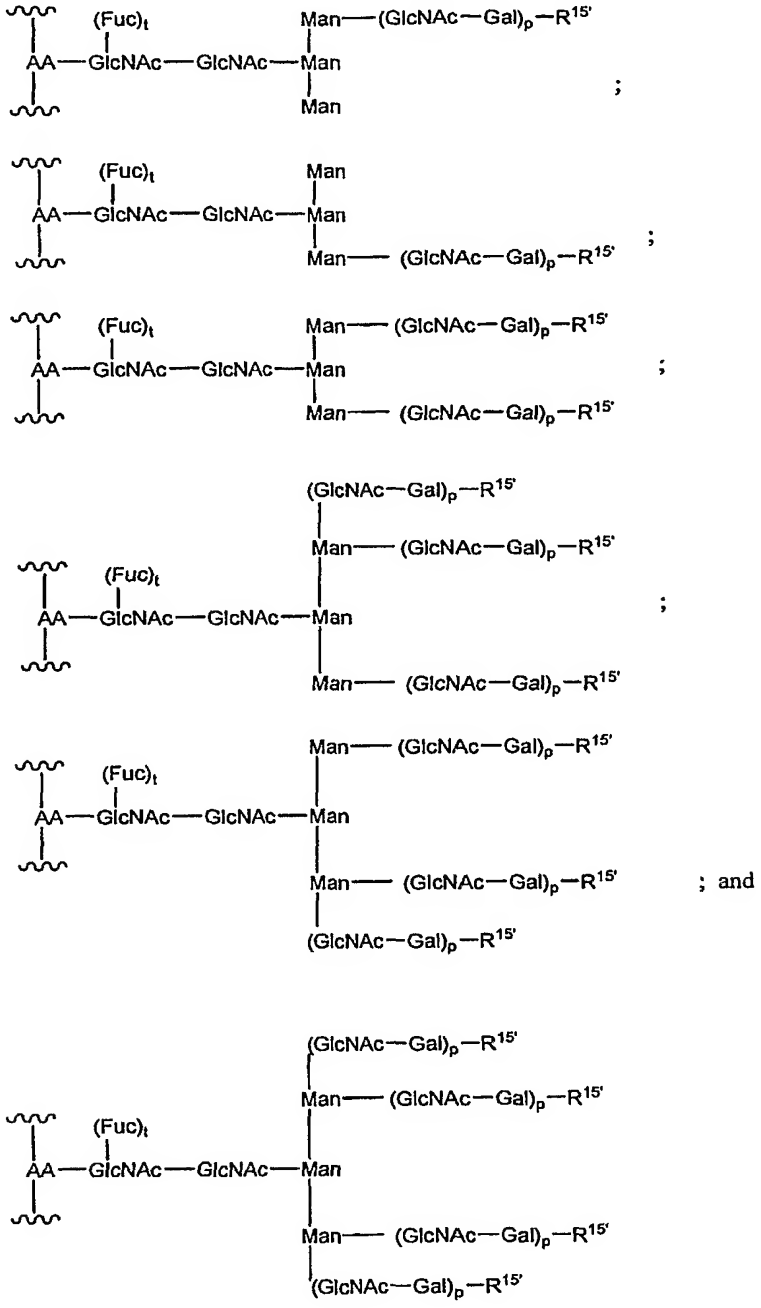
[0210] In yet another embodiment, the glycosyl linking group has the formula:



;

in which the index p represents and integer from 1 to 10; and a is either 0 or 1.

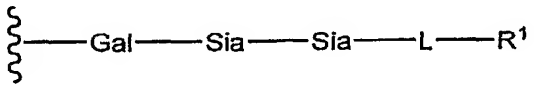
[0211] In an exemplary embodiment, a glycoPEGylated peptide conjugate of the invention selected from the formulae set forth below:



[0212] In the formulae above, the index t is an integer from 0 to 1 and the index p is an integer from 1 to 10. The symbol $R^{15'}$ represents H, OH (*e.g.*, Gal-OH), a sialyl moiety, a sialyl linking group (i.e., sialyl linking group-polymeric modifying group (Sia-L-R^1), or a sialyl moiety to which is bound a polymer modified sialyl moiety (*e.g.*, Sia-Sia-L-R^1) ("Sia-Sia^P")). Exemplary polymer modified saccharyl moieties have a structure according to

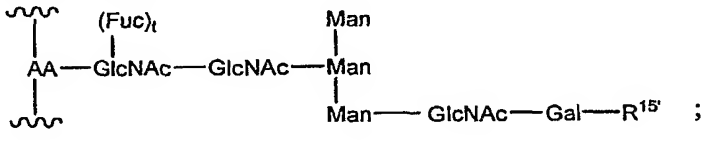
Formulae I and II. An exemplary peptide conjugate of the invention will include at least one glycan having a $R^{15'}$ that includes a structure according to Formulae I or II. The oxygen, with the open valence, of Formulae I and II is preferably attached through a glycosidic linkage to a carbon of a Gal or GalNAc moiety. In a further exemplary embodiment, the oxygen is attached to the carbon at position 3 of a galactose residue. In an exemplary embodiment, the modified sialic acid is linked α2,3-to the galactose residue. In another exemplary embodiment, the sialic acid is linked α2,6-to the galactose residue.

[0213] In an exemplary embodiment, the sialyl linking group is a sialyl moiety to which is bound a polymer modified sialyl moiety (*e.g.*, Sia-Sia-L-R^1) ("Sia-Sia^P"). Here, the glycosyl linking group is linked to a galactosyl moiety through a sialyl moiety:



An exemplary species according to this motif is prepared by conjugating Sia-L-R^1 to a terminal sialic acid of a glycan using an enzyme that forms Sia-Sia bonds, *e.g.*, CST-II, ST8Sia-II, ST8Sia-III and ST8Sia-IV.

[0214] In another exemplary embodiment, the glycans on the peptide conjugates have a formula that is selected from the group:



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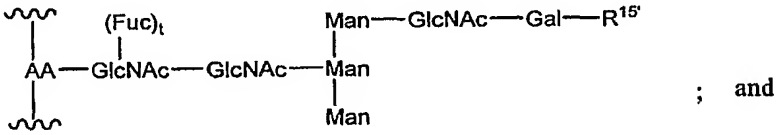
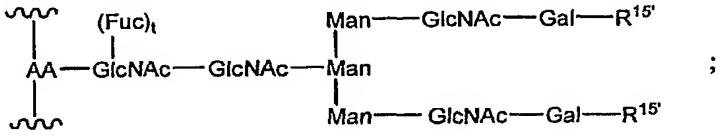


; and

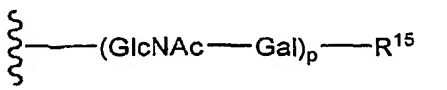


;

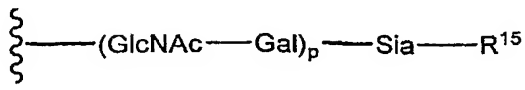
and combinations thereof.

[0215] In each of the formulae above, $R^{15'}$ is as discussed above. Moreover, an exemplary peptide conjugate of the invention will include at least one glycan with an R^{15} moiety having a structure according to Formulae I or II.

[0216] In another exemplary embodiment, the glycosyl linking group comprises at least one glycosyl linking group having the formula:

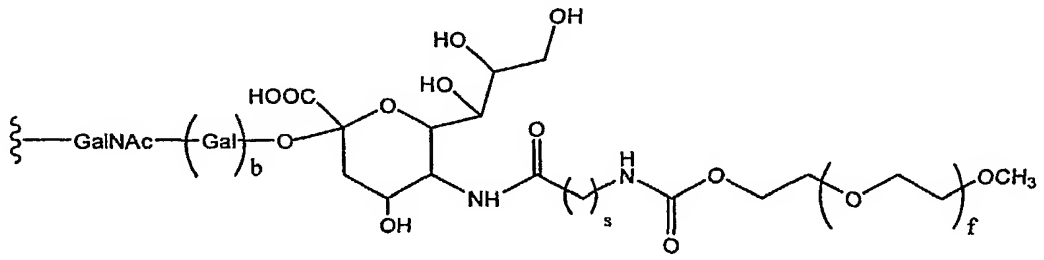


; and



wherein R^{15} is said sialyl linking group; and the index p is an integer selected from 1 to 10.

[0217] In an exemplary embodiment, the glycosyl linking moiety has the formula:



in which b is an integer from 0 to 1. The index s represents an integer from 1 to 10; and the index f represents an integer from 1 to 2500.

[0218] In an exemplary embodiment, the polymeric modifying group is PEG. In another exemplary embodiment, the PEG moiety has a molecular weight of about 20 kDa. In another exemplary embodiment, the PEG moiety has a molecular weight of about 5 kDa. In another exemplary embodiment, the PEG moiety has a molecular weight of about 10 kDa. In another exemplary embodiment, the PEG moiety has a molecular weight of about 40 kDa.

[0219] In an exemplary embodiment, the glycosyl linking group is a linear 10 kDa-PEG-sialyl, and one or two of these glycosyl linking groups are covalently attached to the peptide. In an exemplary embodiment, the glycosyl linking group is a linear 20 kDa-PEG-sialyl, and one or two of these glycosyl linking groups are covalently attached to the peptide. In an exemplary embodiment, the glycosyl linking group is a linear 5 kDa-PEG-sialyl, and one, two or three of these glycosyl linking groups are covalently attached to the peptide. In an exemplary embodiment, the glycosyl linking group is a linear 40 kDa-PEG-sialyl, and one or two of these glycosyl linking groups are covalently attached to the peptide.

Modifying Groups

[0220] The peptide conjugates of the invention comprise a modifying group. This group can be covalently attached to a FGF peptide through an amino acid or a glycosyl linking group. "Modifying groups" can encompass a variety of structures including targeting moieties, therapeutic moieties, biomolecules. Additionally, "modifying groups" include

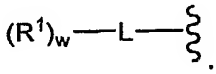
polymeric modifying groups, which are polymers which can alter a property of the peptide such as its bioavailability or its half-life in the body.

[0221] In an exemplary embodiment, the modifying group is a targeting agent that localizes selectively in a particular tissue due to the presence of a targeting agent as a component of the conjugate. In an exemplary embodiment, the targeting agent is a protein. Exemplary proteins include transferrin (brain, blood pool), HS-glycoprotein (bone, brain, blood pool), antibodies (brain, tissue with antibody-specific antigen, blood pool), coagulation factors V-XII (damaged tissue, clots, cancer, blood pool), serum proteins, *e.g.*, α-acid glycoprotein, fetuin, α-fetal protein (brain, blood pool), β2-glycoprotein (liver, atherosclerosis plaques, brain, blood pool), G-CSF, GM-CSF, M-CSF, and EPO (immune stimulation, cancers, blood pool, red blood cell overproduction, neuroprotection), albumin (increase in half-life), and lipoprotein E.

[0222] For the purposes of convenience, the modifying groups in the remainder of this section will be largely based on polymeric modifying groups such as water soluble and water insoluble polymers. However, one of skill in the art will recognize that other modifying groups, such as targeting moieties, therapeutic moieties and biomolecules, could be used in place of the polymeric modifying groups.

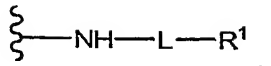
Linkers of the Modifying Groups

[0223] The linkers of the modifying group serve to attach the modifying group (ie polymeric modifying groups, targeting moieties, therapeutic moieties and biomolecules) to the peptide. In an exemplary embodiment, the polymeric modifying group is bound to a glycosyl linking group, generally through a heteroatom, e.g, nitrogen, on the core through a linker, L, as shown below:

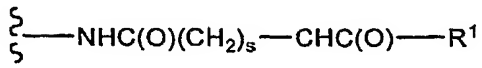


R^1 is the polymeric moiety and L is selected from a bond and a linking group. The index w represents an integer selected from 1-6, preferably 1-3 and more preferably 1-2. Exemplary linking groups include substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl moieties and sialic acid. An exemplary component of the linker is an acyl moiety.

[0224] An exemplary compound according to the invention has a structure according to Formulae I or II above, in which at least one of R^2, R^3, R^4, R^5, R^6 or $R^{6'}$ has the formula:

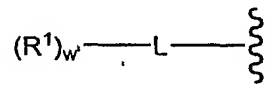


[0225] In another example according to this embodiment at least one of R^2, R^3, R^4, R^5, R^6 or $R^{6'}$ has the formula:



in which s is an integer from 0 to 20 and R^1 is a linear polymeric modifying moiety.

[0226] In an exemplary embodiment, the polymeric modifying group -linker construct is a branched structure that includes two or more polymeric chains attached to central moiety. In this embodiment, the construct has the formula:



in which R^1 and L are as discussed above and w' is an integer from 2 to 6, preferably from 2 to 4 and more preferably from 2 to 3.

[0227] When L is a bond it is formed between a reactive functional group on a precursor of R^1 and a reactive functional group of complementary reactivity on the saccharyl core. When L is a non-zero order linker, a precursor of L can be in place on the glycosyl moiety prior to reaction with the R^1 precursor. Alternatively, the precursors of R^1 and L can be incorporated into a preformed cassette that is subsequently attached to the glycosyl moiety. As set forth herein, the selection and preparation of precursors with appropriate reactive functional groups is within the ability of those skilled in the art. Moreover, coupling the precursors proceeds by chemistry that is well understood in the art.

[0228] In an exemplary embodiment, L is a linking group that is formed from an amino acid, or small peptide (*e.g.*, 1-4 amino acid residues) providing a modified sugar in which the polymeric modifying group is attached through a substituted alkyl linker. Exemplary linkers include glycine, lysine, serine and cysteine. The PEG moiety can be attached to the amine moiety of the linker through an amide or urethane bond. The PEG is linked to the sulfur or oxygen atoms of cysteine and serine through thioether or ether bonds, respectively.

[0229] In an exemplary embodiment, R^5 includes the polymeric modifying group. In another exemplary embodiment, R^5 includes both the polymeric modifying group and a linker, L, joining the modifying group to the remainder of the molecule. As discussed above,

L can be a linear or branched structure. Similarly, the polymeric modifying group can be branched or linear.

Water-Soluble Polymers

[0230] Many water-soluble polymers are known to those of skill in the art and are useful in practicing the present invention. The term water-soluble polymer encompasses species such as saccharides (*e.g.*, dextran, amylose, hyalouronic acid, poly(sialic acid), heparans, heparins, etc.); poly (amino acids), *e.g.*, poly(aspartic acid) and poly(glutamic acid); nucleic acids; synthetic polymers (*e.g.*, poly(acrylic acid), poly(ethers), *e.g.*, poly(ethylene glycol); peptides, proteins, and the like. The present invention may be practiced with any water-soluble polymer with the sole limitation that the polymer must include a point at which the remainder of the conjugate can be attached.

[0231] Methods for activation of polymers can also be found in WO 94/17039, U.S. Pat. No. 5,324,844, WO 94/18247, WO 94/04193, U.S. Pat. No. 5,219,564, U.S. Pat. No. 5,122,614, WO 90/13540, U.S. Pat. No. 5,281,698, and more WO 93/15189, and for conjugation between activated polymers and peptides, *e.g.* Coagulation Factor VIII (WO 94/15625), hemoglobin (WO 94/09027), oxygen carrying molecule (U.S. Pat. No. 4,412,989), ribonuclease and superoxide dismutase (Veronese *at al., App. Biochem. Biotech.* **11**: 141-45 (1985)).

[0232] Exemplary water-soluble polymers are those in which a substantial proportion of the polymer molecules in a sample of the polymer are of approximately the same molecular weight; such polymers are "homodisperse."

[0233] The present invention is further illustrated by reference to a poly(ethylene glycol) conjugate. Several reviews and monographs on the functionalization and conjugation of PEG are available. *See,* for example, Harris, M*acronol. Chem. Phys.* **C25**: 325-373 (1985); Scouten, *Methods in Enzymology* **135**: 30-65 (1987); Wong *et al.*, *Enzyme Microb. Technol.* **14**: 866-874 (1992); Delgado *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems* **9**: 249-304 (1992); Zalipsky, *Bioconjugate Chem.* **6**: 150-165 (1995); and Bhadra, *et al.*, *Pharmazie*, **57**:5-29 (2002). Routes for preparing reactive PEG molecules and forming conjugates using the reactive molecules are known in the art. For example, U.S. Patent No. 5,672,662 discloses a water soluble and isolatable conjugate of an active ester of a polymer acid selected from linear or branched poly(alkylene oxides), poly(oxyethylated polyols), poly(olefinic alcohols), and poly(acrylomorpholine).

[0234] U.S. Patent No. 6,376,604 sets forth a method for preparing a water-soluble 1-benzotriazolylcarbonate ester of a water-soluble and non-peptidic polymer by reacting a terminal hydroxyl of the polymer with di(1-benzotriazoyl)carbonate in an organic solvent. The active ester is used to form conjugates with a biologically active agent such as a protein or peptide.

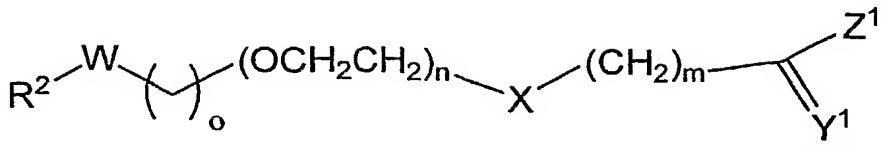
[0235] WO 99/45964 describes a conjugate comprising a biologically active agent and an activated water soluble polymer comprising a polymer backbone having at least one terminus linked to the polymer backbone through a stable linkage, wherein at least one terminus comprises a branching moiety having proximal reactive groups linked to the branching moiety, in which the biologically active agent is linked to at least one of the proximal reactive groups. Other branched poly(ethylene glycols) are described in WO 96/21469, U.S. Patent No. 5,932,462 describes a conjugate formed with a branched PEG molecule that includes a branched terminus that includes reactive functional groups. The free reactive groups are available to react with a biologically active species, such as a protein or peptide, forming conjugates between the poly(ethylene glycol) and the biologically active species. U.S. Patent No. 5,446,090 describes a bifunctional PEG linker and its use in forming conjugates having a peptide at each of the PEG linker termini.

[0236] Conjugates that include degradable PEG linkages are described in WO 99/34833; and WO 99/14259, as well as in U.S. Patent No. 6,348,558. Such degradable linkages are applicable in the present invention.

[0237] The art-recognized methods of polymer activation set forth above are of use in the context of the present invention in the formation of the branched polymers set forth herein and also for the conjugation of these branched polymers to other species, *e.g.*, sugars, sugar nucleotides and the like.

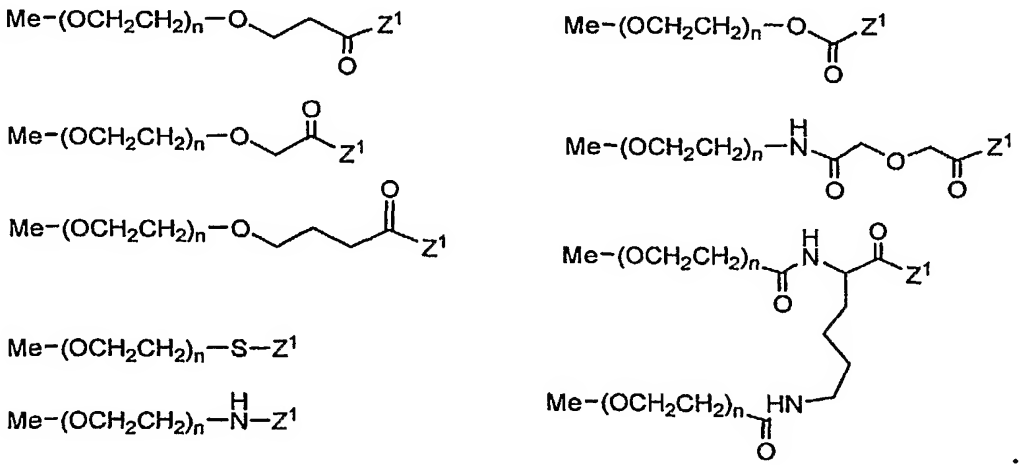
[0238] An exemplary water-soluble polymer is poly(ethylene glycol), *e.g.*, methoxy-poly(ethylene glycol). The poly(ethylene glycol) used in the present invention is not restricted to any particular form or molecular weight range. For unbranched poly(ethylene glycol) molecules the molecular weight is preferably between 500 and 100,000. A molecular weight of 2000-60,000 is preferably used and preferably of from about 5,000 to about 40,000.

[0239] In an examplary embodiment, poly(ethylene glycol) molecules of the invention include, but are not limited to, those species set forth below.



in which R^2 is H, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted heteroalkyl, *e.g.*, acetal, OHC-, H_2N-CH_2CH_2-, HS-CH_2CH_2-, and-$(CH_2)_qC(Y^1)Z^2$; -sugar-nucleotide, or protein. The index "n" represents an integer from 1 to 2500. The indeces m, o, and q independently represent integers from 0 to 20. The symbol Z represents OH, NH_2, halogen, S-R^3, the alcohol portion of activated esters, -$(CH_2)_pC(Y^2)V$, -$(CH_2)_pU(CH_2)_sC(Y^2)_v$, sugar-nucleotide, protein, and leaving groups, *e.g.*, imidazole, p-nitrophenyl, HOBT, tetrazole, halide. The symbols X, Y^1, Y^2, W, U independently represent the moieties O, S, N-R^4. The symbol V represents OH, NH_2, halogen, S-R^5, the alcohol component of activated esters, the amine component of activated amides, sugar-nucleotides, and proteins. The indeces p, s and v are members independently selected from the integers from 0 to 20. The symbols R^3, R^4 and R^5 independently represent H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heterocycloalkyl and substituted or unsubstituted heteroaryl.

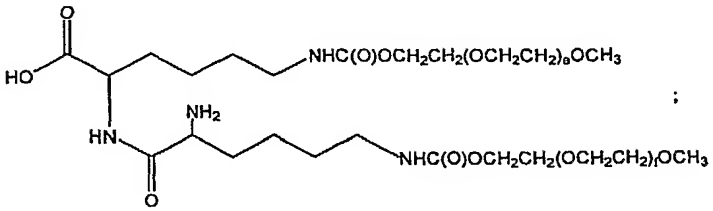
[0240] In other exemplary embodiments, the poly(ethylene glycol) molecule is selected from the following:



[0241] In another embodiment the poly(ethylene glycol) is a branched PEG having more than one PEG moiety attached. Examples of branched PEGs are described in U.S. Pat. No. 5,932,462; U.S. Pat. No. 5,342,940; U.S. Pat. No. 5,643,575; U.S. Pat. No. 5,919,455; U.S.

Pat. No. 6,113,906; U.S. Pat. No. 5,183,660; WO 02/09766; Kodera Y., *Bioconjugate Chemistry* **5**: 283-288 (1994); and Yamasaki et al., *Agric. Biol. Chem.*, **52**: 2125-2127, 1998. In a preferred embodiment the molecular weight of each poly(ethylene glycol) of the branched PEG is less than or equal to 40,000 daltons.

[0242] Representative polymeric modifying moieties include structures that are based on side chain-containing amino acids, *e.g.*, serine, cysteine, lysine, and small peptides, *e.g.*, lys-lys. Exemplary structures include:



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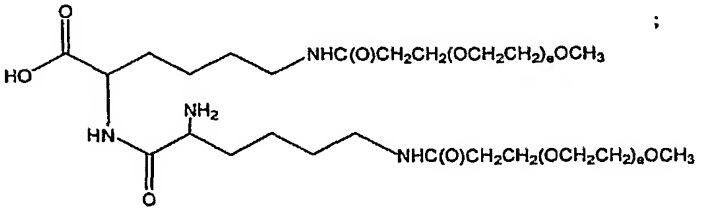

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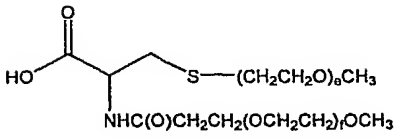

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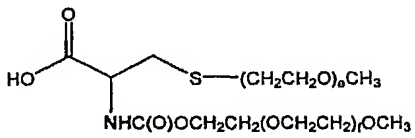

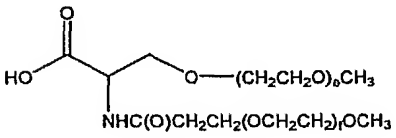

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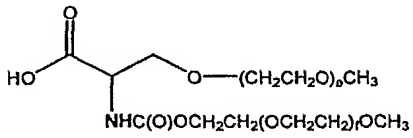
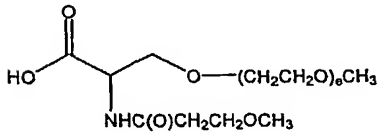
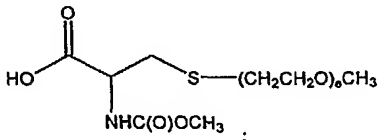


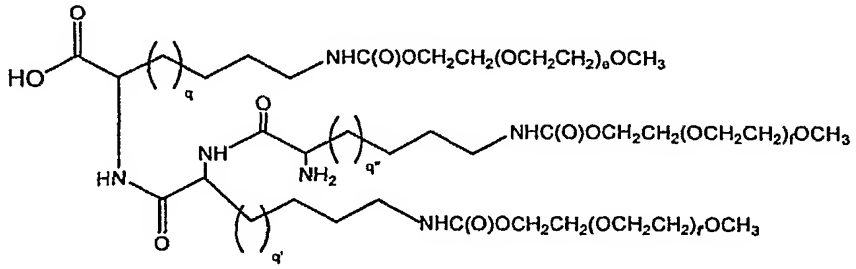




; and

Those of skill will appreciate that the free amine in the di-lysine structures can also be pegylated through an amide or urethane bond with a PEG moiety.

[0243] In yet another embodiment, the polymeric modifying moiety is a branched PEG moiety that is based upon a tri-lysine peptide. The tri-lysine can be mono-, di-, tri-, or tetra-PEG-ylated. Exemplary species according to this embodiment have the formulae:



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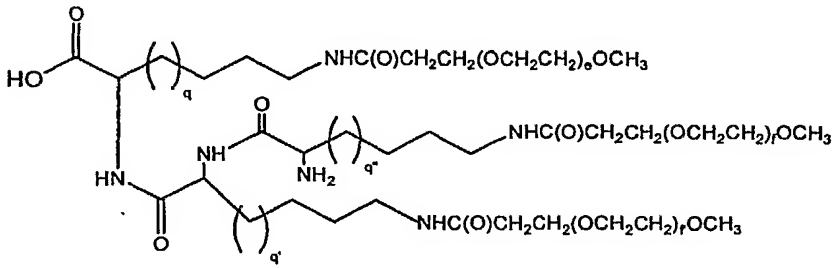
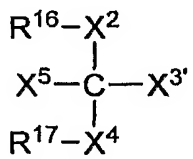


in which the indices e, f and f' are independently selected integers from 1 to 2500; and the indices q, q' and q" are independently selected integers from 1 to 20.

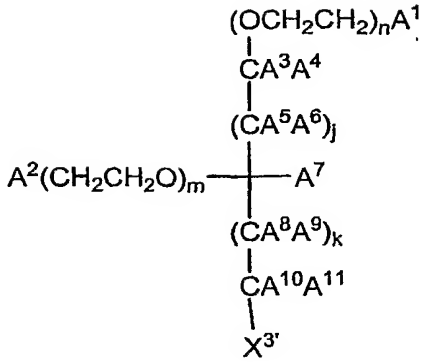
[0244] As will be apparent to those of skill, the branched polymers of use in the invention include variations on the themes set forth above. For example the di-lysine-PEG conjugate shown above can include three polymeric subunits, the third bonded to the α-amine shown as unmodified in the structure above. Similarly, the use of a tri-lysine functionalized with three or four polymeric subunits labeled with the polymeric modifying moiety in a desired manner is within the scope of the invention.

[0245] As discussed herein, the PEG of use in the conjugates of the invention can be linear or branched. An exemplary precursor of use to form the branched PEG containing peptide conjugates according to this embodiment of the invention has the formula:



(III).

Another exemplary precursor of use to form the branched PEG containing peptide conjugates according to this embodiment of the invention has the formula:



in which the indices m and n are integers independently selected from 0 to 5000. A^1, A^2, A^3, A^4, A^5, A^6, A^7, A^8, A^9, A^{10} and A^{11} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, $-NA^{12}A^{13}$, $-OA^{12}$ and $-SiA^{12}A^{13}$. A^{12} and A^{13} are members independently selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, and substituted or unsubstituted heteroaryl.

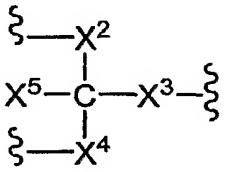
[0246] The branched polymer species according to this formula are essentially pure water-soluble polymers. $X^{3'}$ is a moiety that includes an ionizable (*e.g.*, OH, COOH, H_2PO_4, HSO_3, NH_2, and salts thereof, etc.) or other reactive functional group, *e.g.*, *infra*. C is carbon. X^5, R^{16} and R^{17} are independently selected from non-reactive groups (*e.g.*, H, unsubstituted alkyl, unsubstituted heteroalkyl) and polymeric arms (*e.g.*, PEG). X^2 and X^4 are linkage fragments that are preferably essentially non-reactive under physiological conditions, which may be the same or different. An exemplary linker includes neither aromatic nor ester moieties. Alternatively, these linkages can include one or more moiety that is designed to degrade under physiologically relevant conditions, *e.g.*, esters, disulfides, etc. X^2 and X^4 join polymeric arms R^{16} and R^{17} to C. When $X^{3'}$ is reacted with a reactive functional group of complementary reactivity on a linker, sugar or linker-sugar cassette, $X^{3'}$ is converted to a component of linkage fragment X^3.

[0247] Exemplary linkage fragments for X^2, X^3 and X^4 are independently selected and include S, SC(O)NH, HNC(O)S, SC(O)O, O, NH, NHC(O), (O)CNH and NHC(O)O, and OC(O)NH, CH_2S, CH_2O , CH_2CH_2O, CH_2CH_2S, $(CH_2)_oO$, $(CH_2)_oS$ or $(CH_2)_oY'$-PEG wherein, Y' is S, NH, NHC(O), C(O)NH, NHC(O)O, OC(O)NH, or O and o is an integer

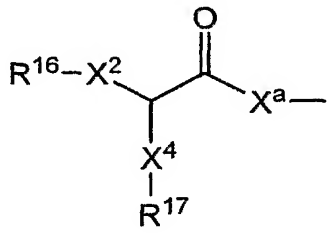
from 1 to 50. In an exemplary embodiment, the linkage fragments X^2 and X^4 are different linkage fragments.

[0248] In an exemplary embodiment, the precursor (Formula III), or an activated derivative thereof, is reacted with, and thereby bound to a sugar, an activated sugar or a sugar nucleotide through a reaction between $X^{3'}$ and a group of complementary reactivity on the sugar moiety, *e.g.*, an amine. Alternatively, $X^{3'}$ reacts with a reactive functional group on a precursor to linker, L. One or more of R^2, R^3, R^4, R^5, R^6 or $R^{6'}$ of Formulae I and II can include the branched polymeric modifying moiety, or this moiety bound through L.

[0249] In an exemplary embodiment, the moiety:



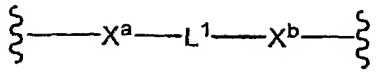
is the linker arm, L. In this embodiment, an exemplary linker is derived from a natural or unnatural amino acid, amino acid analogue or amino acid mimetic, or a small peptide formed from one or more such species. For example, certain branched polymers found in the compounds of the invention have the formula:



(IV)

[0250] X^a is a linkage fragment that is formed by the reaction of a reactive functional group, *e.g.*, $X^{3'}$, on a precursor of the branched polymeric modifying moiety and a reactive functional group on the sugar moiety, or a precursor to a linker. For example, when $X^{3'}$ is a carboxylic acid, it can be activated and bound directly to an amine group pendent from an amino-saccharide (*e.g.*, Sia, $GalNH_2$, $GlcNH_2$, $ManNH_2$, etc.), forming a X^a that is an amide. Additional exemplary reactive functional groups and activated precursors are described hereinbelow. The index c represents an integer from 1 to 10. The other symbols have the same identity as those discussed above.

[0251] In another exemplary embodiment, X^a is a linking moiety formed with another linker:

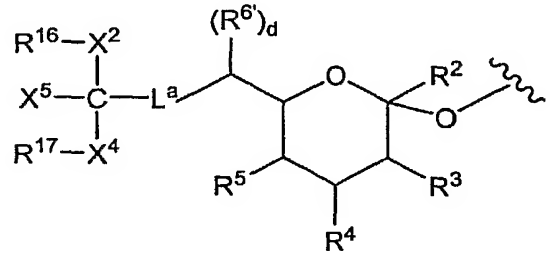


in which X^b is a second linkage fragment and is independently selected from those groups set forth for X^a, and, similar to L, L^1 is a bond, substituted or unsubstituted alkyl or substituted or unsubstituted heteroalkyl.

[0252] Exemplary species for X^a and X^b include S, SC(O)NH, HNC(O)S, SC(O)O, O, NH, NHC(O), C(O)NH and NHC(O)O, and OC(O)NH.

[0253] In another exemplary embodiment, X^4 is a peptide bond to R^{17}, which is an amino acid, di-peptide (*e.g.*,, Lys-Lys) or tri-peptide (*e.g.*, Lys-Lys-Lys) in which the alpha-amine moiety(ies) and/or side chain heteroatom(s) are modified with a polymeric modifying moiety.

[0254] In a further exemplary embodiment, the peptide conjugates of the invention include a moiety, *e.g.*, an R^{15} moiety that has a formula that is selected from:



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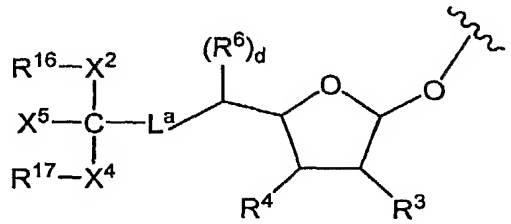
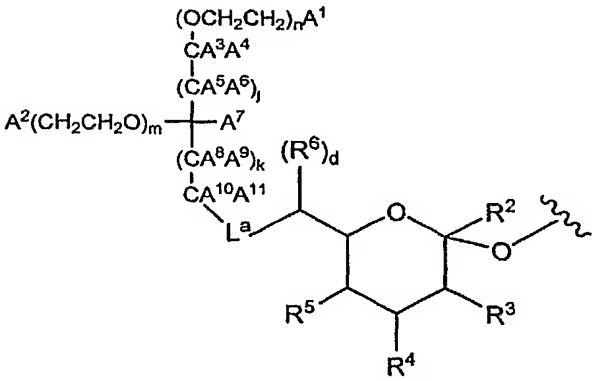


VI



Va

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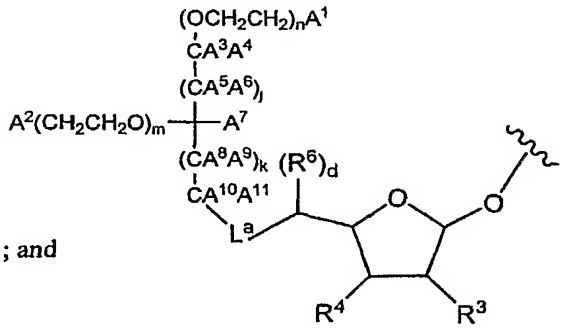
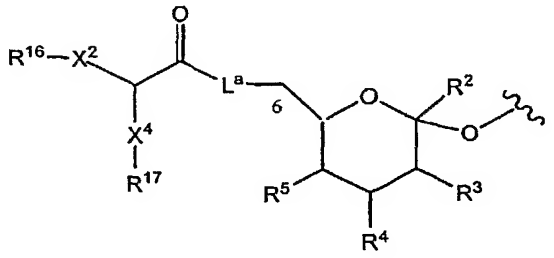


VIa

in which the identity of the radicals represented by the various symbols is the same as that discussed hereinabove. L^a is a bond or a linker as discussed above for L and L^1, *e.g.*, substituted or unsubstituted alkyl or substituted or unsubstituted heteroalkyl moiety. In an exemplary embodiment, L^a is a moiety of the side chain of sialic acid that is functionalized with the polymeric modifying moiety as shown. Exemplary L^a moieties include substituted or unsubstituted alkyl chains that include one or more OH or NH_2.

[0255] In yet another exemplary embodiment, the invention provides peptide conjugates having a moiety, *e.g.*, an R^{15} moiety with formula:



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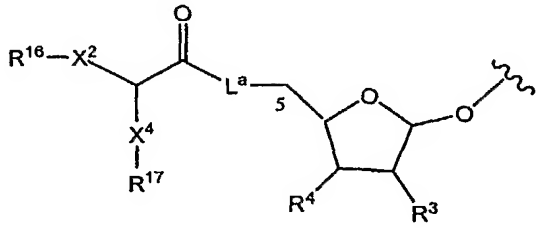
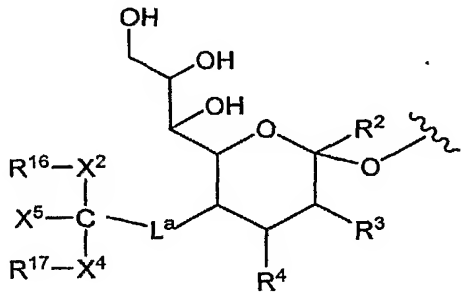


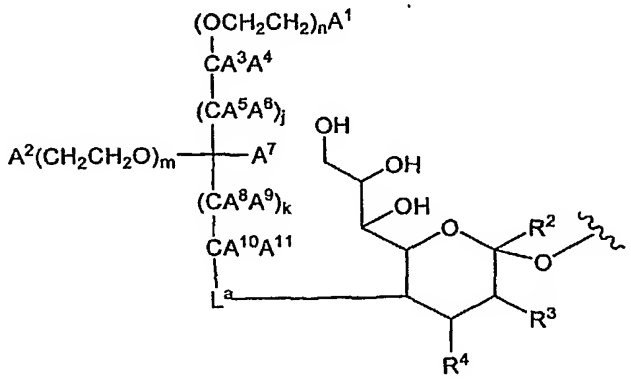
VII VIII

The identity of the radicals represented by the various symbols is the same as that discussed hereinabove. As those of skill will appreciate, the linker arm in Formulae VII and VIII is equally applicable to other modified sugars set forth herein. In exemplary embodiment, the species of Formulae VII and VIII are the R^{15} moieties attached to the glycan structures set forth herein.

[0256] In yet another exemplary embodiment, the Factor VII or Factor VIIa peptide conjugate includes a R^{15} moiety with a formula which is a member selected from:



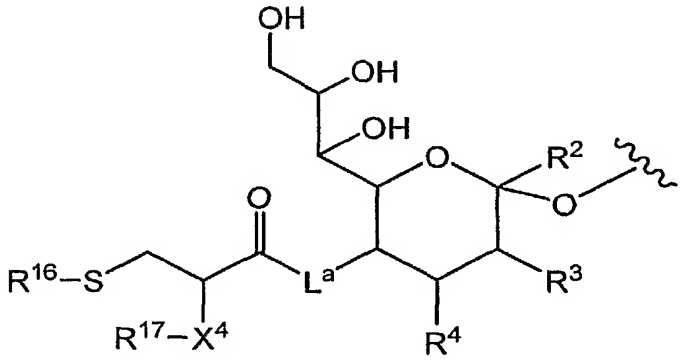
and



in which the identities of the radicals are as discussed above. An exemplary species for L^a is $-(CH_2)_jC(O)NH(CH_2)_hC(O)NH-$, in which the indices h and j are independently selected

integers from 0 to 10. A further exemplary species is –C(O)NH-. The indices m and n are integers independently selected from 0 to 5000. A^1, A^2, A^3, A^4, A^5, A^6, A^7, A^8, A^9, A^{10} and A^{11} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, $-NA^{12}A^{13}$, $-OA^{12}$ and $-SiA^{12}A^{13}$. A^{12} and A^{13} are members independently selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, and substituted or unsubstituted heteroaryl.

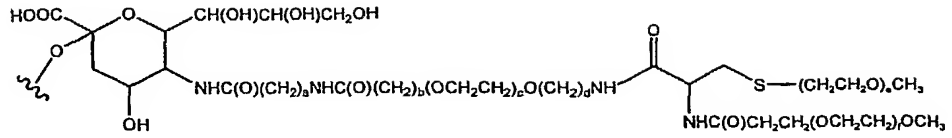
[0257] In an exemplary embodiment, the glycosyl linking group has a structure according to the following formula:

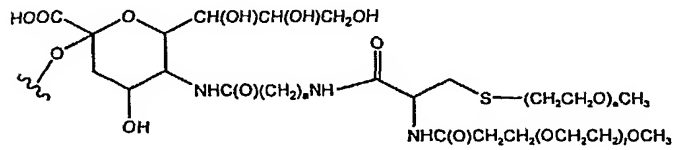


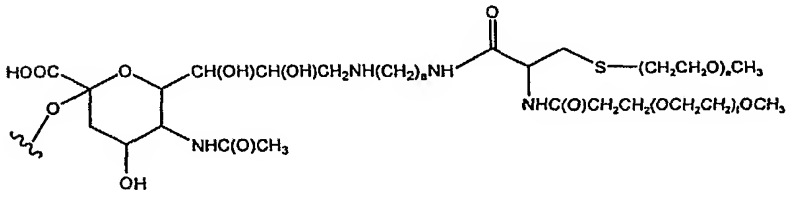
[0258] The embodiments of the invention set forth above are further exemplified by reference to species in which the polymer is a water-soluble polymer, particularly poly(ethylene glycol) ("PEG"), *e.g.*, methoxy-poly(ethylene glycol). Those of skill will appreciate that the focus in the sections that follow is for clarity of illustration and the various motifs set forth using PEG as an exemplary polymer are equally applicable to species in which a polymer other than PEG is utilized.

[0259] PEG of any molecular weight, *e.g.*, 1 kDa, 2 kDa, 5 kDa, 10 kDa, 15 kDa, 20 kDa, 25 kDa, 30 kDa, 35 kDa, 40 kDa and 45 kDa is of use in the present invention.

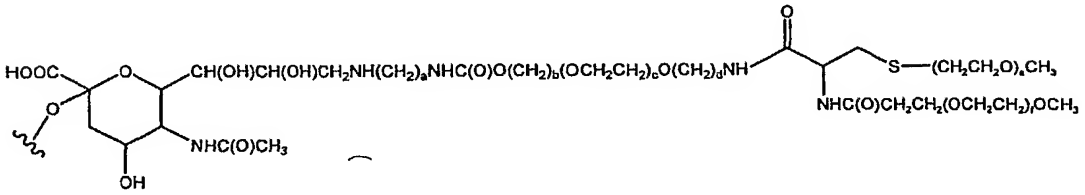
[0260] In an exemplary embodiment, the R^{15} moiety has a formula that is a member selected from the group:





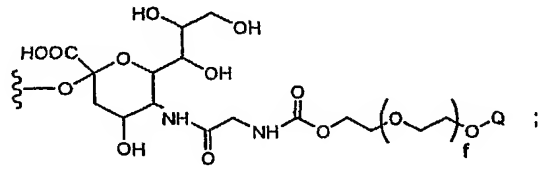


and



In each of the structures above, the linker fragment $-NH(CH_2)_a-$ can be present or absent.

[0261] In other exemplary embodiments, the peptide conjugate includes an R^{15} moiety selected from the group:



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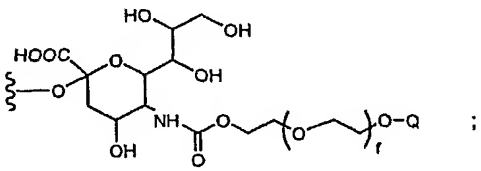

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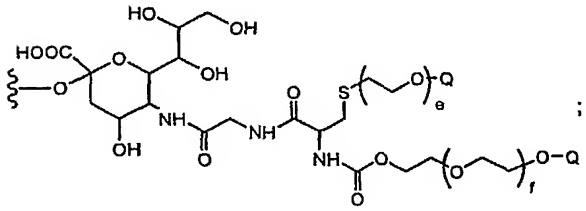

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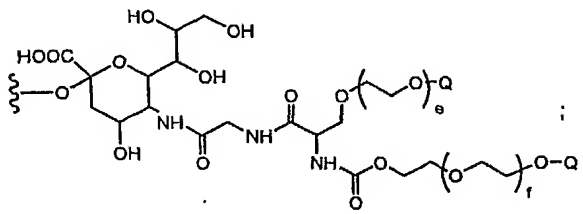

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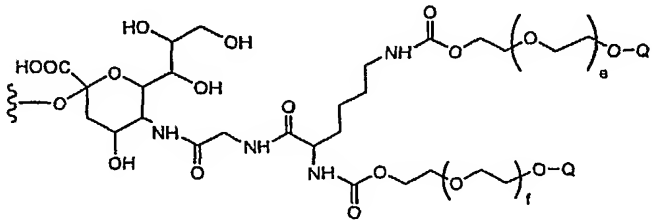

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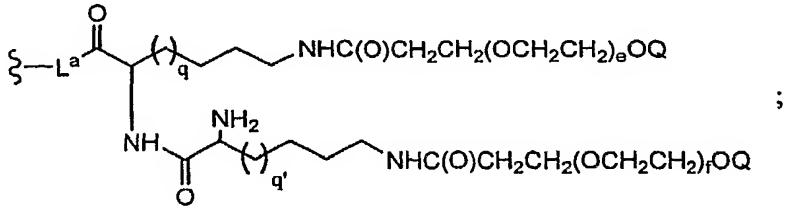
[0262] In each of the formulae above, the indices e and f are independently selected from the integers from 1 to 2500. In further exemplary embodiments, e and f are selected to provide a PEG moiety that is about 1 kDa, 2 kDa, 5 kDa, 10 kDa, 15 kDa, 20 kDa, 25 kDa, 30 kDa, 35 kDa, 40 kDa and 45 kDa. The symbol Q represents substituted or unsubstituted alkyl (*e.g.*, C_1-C_6 alkyl, *e.g.*, methyl), substituted or unsubstituted heteroalkyl or H.

[0263] Other branched polymers have structures based on di-lysine (Lys-Lys) peptides, *e.g.*:



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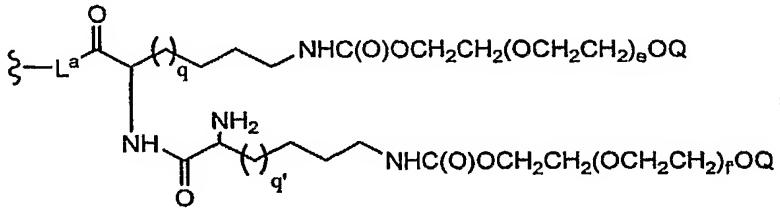

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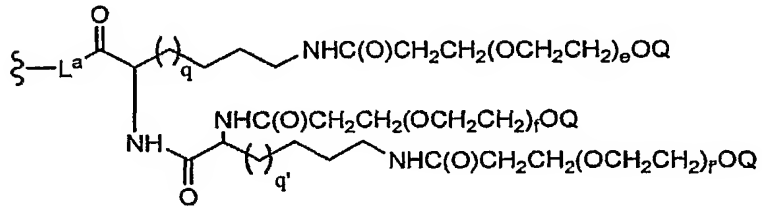
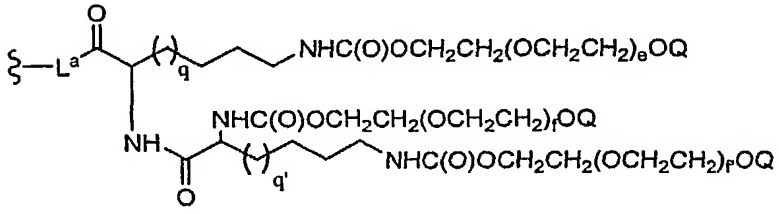
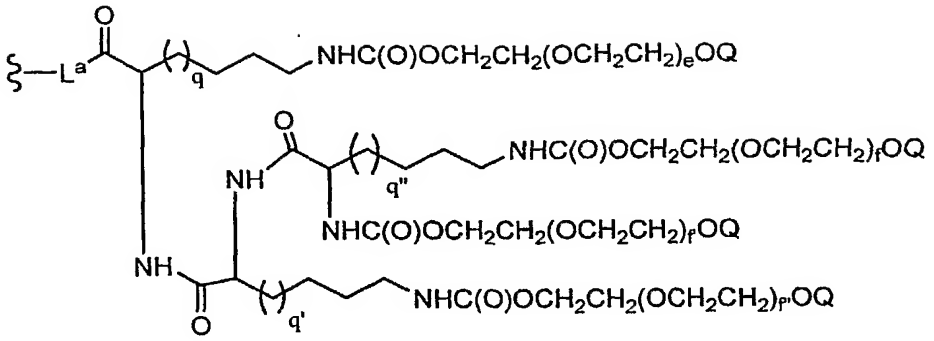


; and



;

and tri-lysine peptides (Lys-Lys-Lys), *e.g.*:



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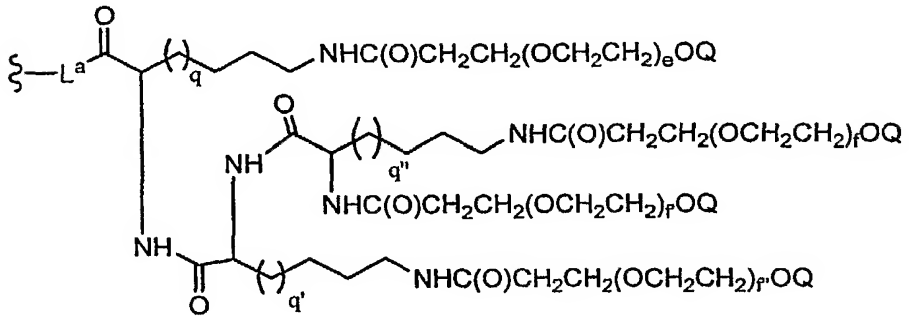
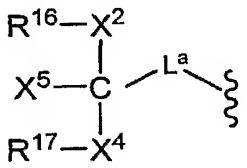
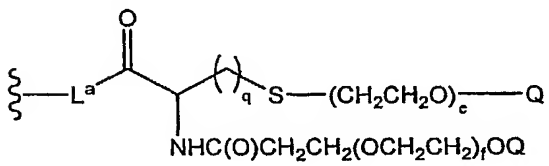


In each of the figures above, the indices e, f, f' and f" represent integers independently selected from 1 to 2500. The indices q, q' and q" represent integers independently selected from 1 to 20.

[0264] In another exemplary embodiment, the modifying group:



has a formula that is a member selected from:



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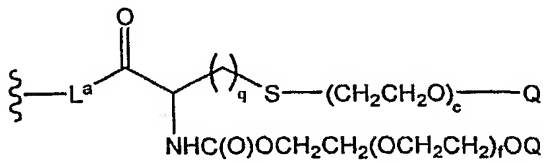
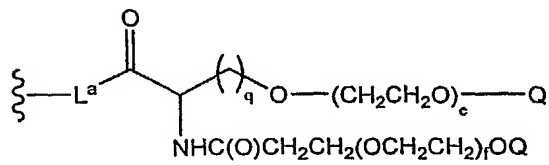
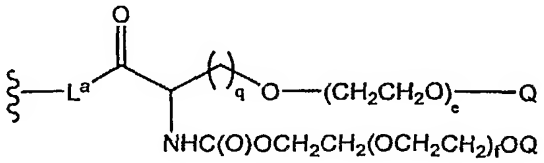


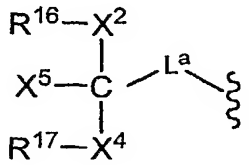


; and

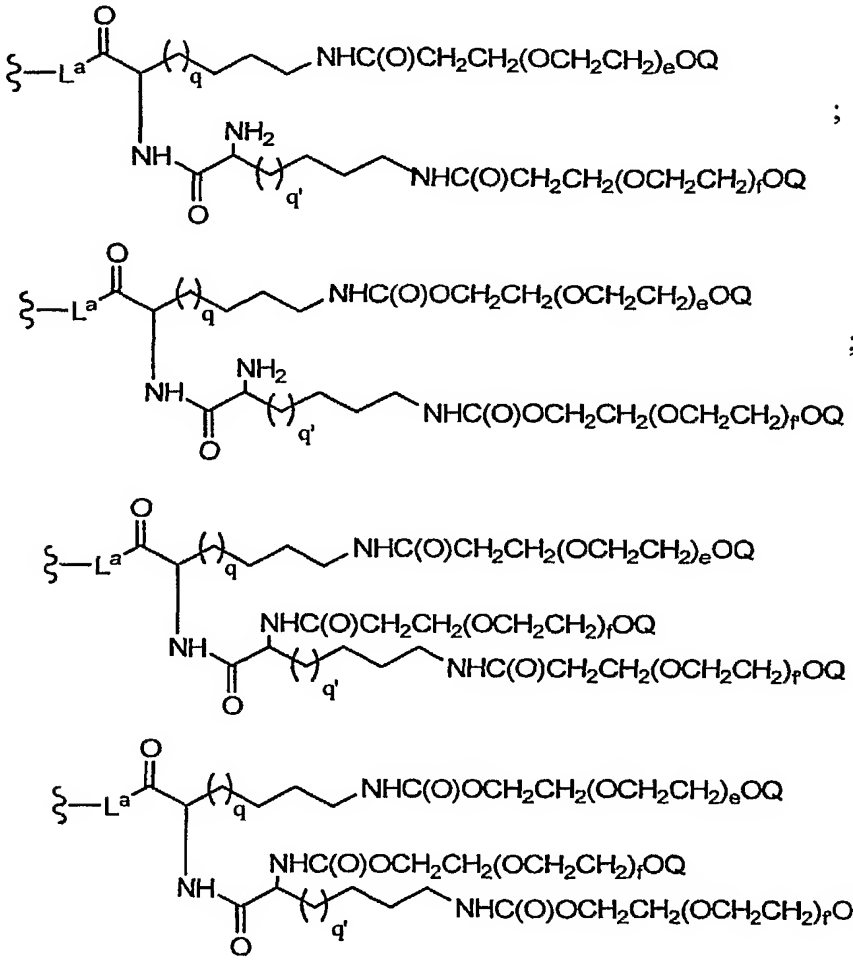


wherein Q is a member selected from H and substituted or unsubstituted C_1-C_6 alkyl. The indices e and f are integers independently selected from 1 to 2500, and the index q is an integer selected from 0 to 20.

[0265] In another exemplary embodiment, the modifying group:

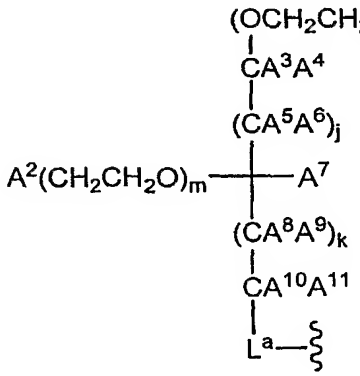


has a formula that is a member selected from:



wherein Q is a member selected from H and substituted or unsubstituted C_1-C_6 alkyl. The indices e, f and f' are integers independently selected from 1 to 2500, and q and q' are integers independently selected from 1 to 20.

[0266] In another exemplary embodiment, the branched polymer has a structure according to the following formula:

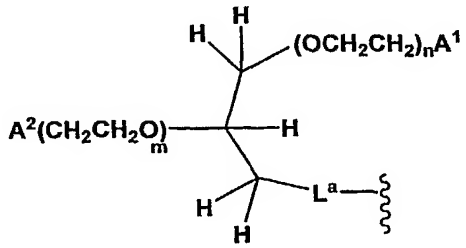


(IIIa)

in which the indices m and n are integers independently selected from 0 to 5000. A^1, A^2, A^3, A^4, A^5, A^6, A^7, A^8, A^9, A^{10} and A^{11} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, $-NA^{12}A^{13}$, $-OA^{12}$ and $-SiA^{12}A^{13}$. A^{12} and A^{13} are members independently selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, and substituted or unsubstituted heteroaryl.

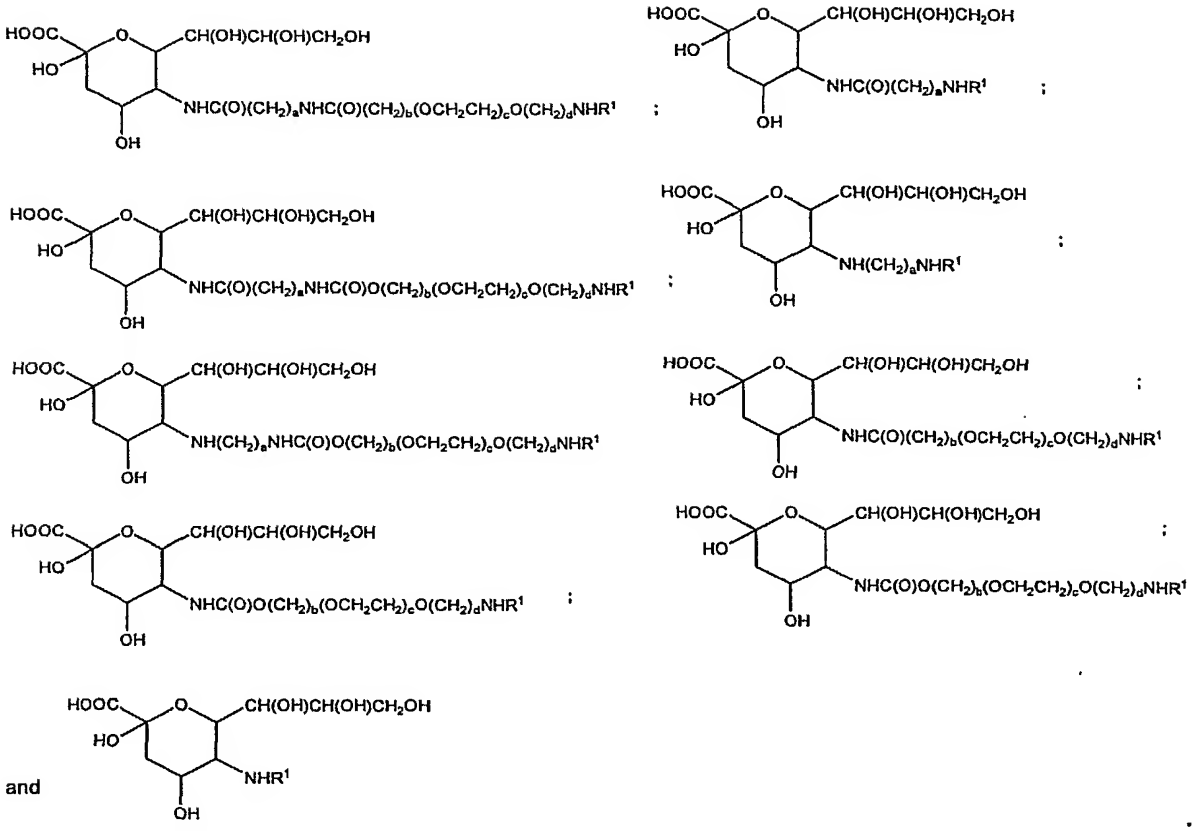
[0267] Formula IIIa is a subset of Formula III. The structures described by Formula IIIa are also encompassed by Formula III.

[0268] In another exemplary embodiment according to the formula above, the branched polymer has a structure according to the following formula:



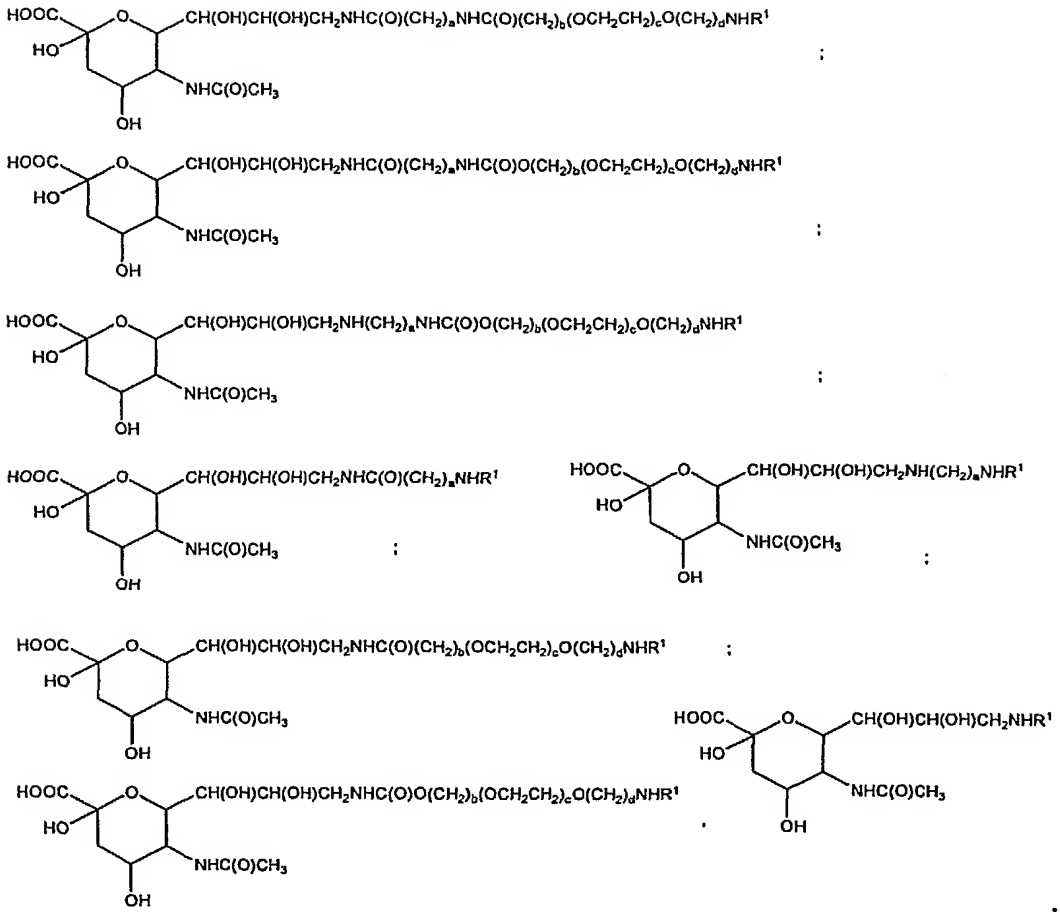
In an exemplary embodiment, A^1 and A^2 are each $-OCH_3$ or H.

[0269] In an illustrative embodiment, the modified sugar is sialic acid and selected modified sugar compounds of use in the invention have the formulae:



The indices a, b and d are integers from 0 to 20. The index c is an integer from 1 to 2500. The structures set forth above can be components of R^{15}.

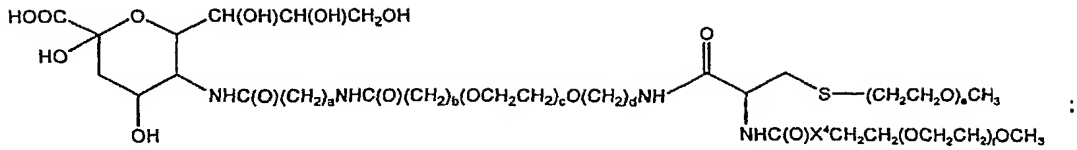
[0270] In another illustrative embodiment, a primary hydroxyl moiety of the sugar is functionalized with the modifying group. For example, the 9-hydroxyl of sialic acid can be converted to the corresponding amine and functionalized to provide a compound according to the invention. Formulae according to this embodiment include:

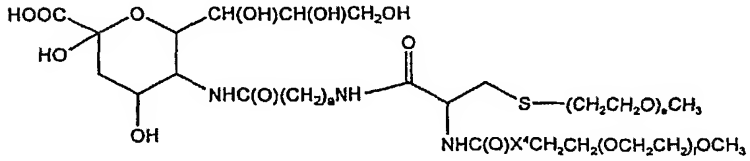


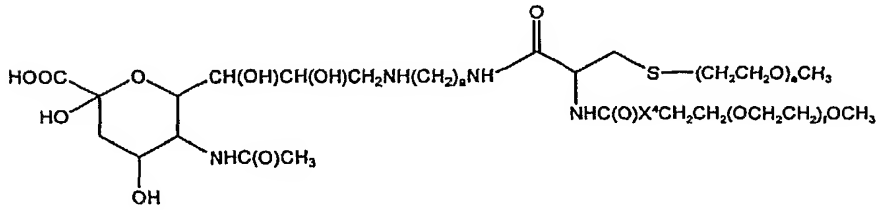
The structures set forth above can be components of R^{15}.

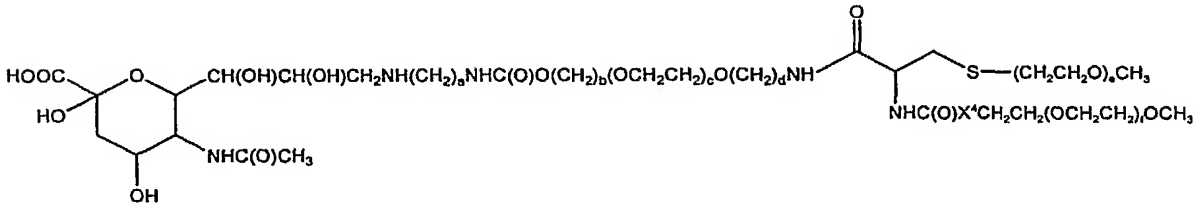
[0271] Although the present invention is exemplified in the preceding sections by reference to PEG, as those of skill will appreciate, an array of polymeric modifying moieties is of use in the compounds and methods set forth herein.

[0272] In selected embodiments, R^1 or L-R^1 is a branched PEG, for example, one of the species set forth above. In an exemplary embodiment, the branched PEG structure is based on a cysteine peptide. Illustrative modified sugars according to this embodiment include:



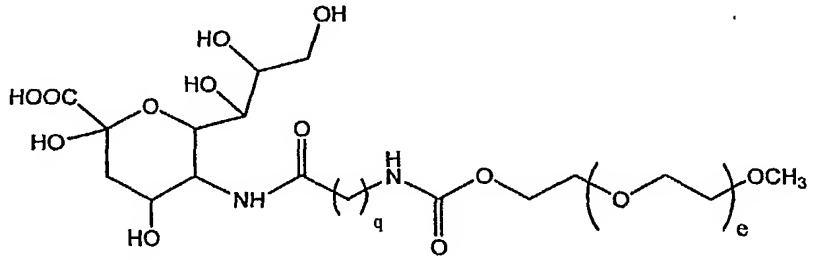






in which X^4 is a bond or O. In each of the structures above, the alkylamine linker $-(CH_2)_aNH-$ can be present or absent. The structures set forth above can be components of $R^{15}/R^{15'}$.

[0273] As discussed herein, the polymer-modified sialic acids of use in the invention may also be linear structures. Thus, the invention provides for conjugates that include a sialic acid moiety derived from a structure such as:

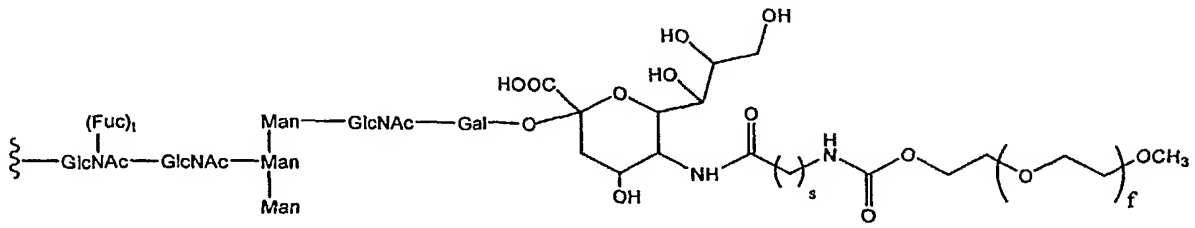


in which the indices q and e are as discussed above.

[0274] Exemplary modified sugars are modified with water-soluble or water-insoluble polymers. Examples of useful polymer are further exemplified below.

[0275] In another exemplary embodiment, the peptide is derived from insect cells, remodeled by adding GlcNAc and Gal to the mannose core and glycopegylated using a sialic

acid bearing a linear PEG moiety, affording a Factor VII or Factor VIIa peptide that comprises at least one moiety having the formula:



in which the index t is an integer from 0 to 1; the index s represents an integer from 1 to 10; and the index f represents an integer from 1 to 2500.

Water-Insoluble Polymers

[0276] In another embodiment, analogous to those discussed above, the modified sugars include a water-insoluble polymer, rather than a water-soluble polymer. The conjugates of the invention may also include one or more water-insoluble polymers. This embodiment of the invention is illustrated by the use of the conjugate as a vehicle with which to deliver a therapeutic peptide in a controlled manner. Polymeric drug delivery systems are known in the art. *See*, for example, Dunn *et al.*, Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991. Those of skill in the art will appreciate that substantially any known drug delivery system is applicable to the conjugates of the present invention.

[0277] The motifs forth above for R^1, L-R^1, R^{15}, $R^{15'}$ and other radicals are equally applicable to water-insoluble polymers, which may be incorporated into the linear and branched structures without limitation utilizing chemistry readily accessible to those of skill in the art. Similarly, the incorporation of these species into any of the modified sugars discussed herein is within the scope of the present invention. Accordingly, the invention provides conjugates containing, and for the use of to prepare such conjugates, sialic acid and other sugar moieties modified with a linear or branched water-insoluble polymers, and activated analogues of the modified sialic acid species (*e.g.*, CMP-Sia-(water insoluble polymer)).

[0278] Representative water-insoluble polymers include, but are not limited to, polyphosphazines, poly(vinyl alcohols), polyamides, polycarbonates, polyalkylenes, polyacrylamides, polyalkylene glycols, polyalkylene oxides, polyalkylene terephthalates, polyvinyl ethers, polyvinyl esters, polyvinyl halides, polyvinylpyrrolidone, polyglycolides,

polysiloxanes, polyurethanes, poly(methyl methacrylate), poly(ethyl methacrylate), poly(butyl methacrylate), poly(isobutyl methacrylate), poly(hexyl methacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate) polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly (ethylene terephthalate), poly(vinyl acetate), polyvinyl chloride, polystyrene, polyvinyl pyrrolidone, pluronics and polyvinylphenol and copolymers thereof.

[0279] Synthetically modified natural polymers of use in conjugates of the invention include, but are not limited to, alkyl celluloses, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, and nitrocelluloses. Particularly preferred members of the broad classes of synthetically modified natural polymers include, but are not limited to, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxymethyl cellulose, cellulose triacetate, cellulose sulfate sodium salt, and polymers of acrylic and methacrylic esters and alginic acid.

[0280] These and the other polymers discussed herein can be readily obtained from commercial sources such as Sigma Chemical Co. (St. Louis, MO.), Polysciences (Warrenton, PA.), Aldrich (Milwaukee, WI.), Fluka (Ronkonkoma, NY), and BioRad (Richmond, CA), or else synthesized from monomers obtained from these suppliers using standard techniques.

[0281] Representative biodegradable polymers of use in the conjugates of the invention include, but are not limited to, polylactides, polyglycolides and copolymers thereof, poly(ethylene terephthalate), poly(butyric acid), poly(valeric acid), poly(lactide-co-caprolactone), poly(lactide-co-glycolide), polyanhydrides, polyorthoesters, blends and copolymers thereof. Of particular use are compositions that form gels, such as those including collagen, pluronics and the like.

[0282] The polymers of use in the invention include "hybrid' polymers that include water-insoluble materials having within at least a portion of their structure, a bioresorbable molecule. An example of such a polymer is one that includes a water-insoluble copolymer, which has a bioresorbable region, a hydrophilic region and a plurality of crosslinkable functional groups per polymer chain.

[0283] For purposes of the present invention, "water-insoluble materials" includes materials that are substantially insoluble in water or water-containing environments. Thus,

although certain regions or segments of the copolymer may be hydrophilic or even water-soluble, the polymer molecule, as a whole, does not to any substantial measure dissolve in water.

[0284] For purposes of the present invention, the term "bioresorbable molecule" includes a region that is capable of being metabolized or broken down and resorbed and/or eliminated through normal excretory routes by the body. Such metabolites or break down products are preferably substantially non-toxic to the body.

[0285] The bioresorbable region may be either hydrophobic or hydrophilic, so long as the copolymer composition as a whole is not rendered water-soluble. Thus, the bioresorbable region is selected based on the preference that the polymer, as a whole, remains water-insoluble. Accordingly, the relative properties, *i.e.*, the kinds of functional groups contained by, and the relative proportions of the bioresorbable region, and the hydrophilic region are selected to ensure that useful bioresorbable compositions remain water-insoluble.

[0286] Exemplary resorbable polymers include, for example, synthetically produced resorbable block copolymers of poly(α-hydroxy-carboxylic acid)/poly(oxyalkylene, (*see*, Cohn *et al.*, U.S. Patent No. 4,826,945). These copolymers are not crosslinked and are water-soluble so that the body can excrete the degraded block copolymer compositions. *See*, Younes *et al.*, *J Biomed. Mater. Res.* **21**: 1301-1316 (1987); and Cohn *et al.*, *J Biomed. Mater. Res.* **22**: 993-1009 (1988).

[0287] Presently preferred bioresorbable polymers include one or more components selected from poly(esters), poly(hydroxy acids), poly(lactones), poly(amides), poly(ester-amides), poly (amino acids), poly(anhydrides), poly(orthoesters), poly(carbonates), poly(phosphazines), poly(phosphoesters), poly(thioesters), polysaccharides and mixtures thereof. More preferably still, the biosresorbable polymer includes a poly(hydroxy) acid component. Of the poly(hydroxy) acids, polylactic acid, polyglycolic acid, polycaproic acid, polybutyric acid, polyvaleric acid and copolymers and mixtures thereof are preferred.

[0288] In addition to forming fragments that are absorbed *in vivo* ("bioresorbed"), preferred polymeric coatings for use in the methods of the invention can also form an excretable and/or metabolizable fragment.

[0289] Higher order copolymers can also be used in the present invention. For example, Casey *et al.*, U.S. Patent No. 4,438,253, which issued on March 20, 1984, discloses tri-block

copolymers produced from the transesterification of poly(glycolic acid) and an hydroxyl-ended poly(alkylene glycol). Such compositions are disclosed for use as resorbable monofilament sutures. The flexibility of such compositions is controlled by the incorporation of an aromatic orthocarbonate, such as tetra-p-tolyl orthocarbonate into the copolymer structure.

[0290] Other polymers based on lactic and/or glycolic acids can also be utilized. For example, Spinu, U.S. Patent No. 5,202,413, which issued on April 13, 1993, discloses biodegradable multi-block copolymers having sequentially ordered blocks of polylactide and/or polyglycolide produced by ring-opening polymerization of lactide and/or glycolide onto either an oligomeric diol or a diamine residue followed by chain extension with a di-functional compound, such as, a diisocyanate, diacylchloride or dichlorosilane.

[0291] Bioresorbable regions of coatings useful in the present invention can be designed to be hydrolytically and/or enzymatically cleavable. For purposes of the present invention, "hydrolytically cleavable" refers to the susceptibility of the copolymer, especially the bioresorbable region, to hydrolysis in water or a water-containing environment. Similarly, "enzymatically cleavable" as used herein refers to the susceptibility of the copolymer, especially the bioresorbable region, to cleavage by endogenous or exogenous enzymes.

[0292] When placed within the body, the hydrophilic region can be processed into excretable and/or metabolizable fragments. Thus, the hydrophilic region can include, for example, polyethers, polyalkylene oxides, polyols, poly(vinyl pyrrolidine), poly(vinyl alcohol), poly(alkyl oxazolines), polysaccharides, carbohydrates, peptides, proteins and copolymers and mixtures thereof. Furthermore, the hydrophilic region can also be, for example, a poly(alkylene) oxide. Such poly(alkylene) oxides can include, for example, poly(ethylene) oxide, poly(propylene) oxide and mixtures and copolymers thereof.

[0293] Polymers that are components of hydrogels are also useful in the present invention. Hydrogels are polymeric materials that are capable of absorbing relatively large quantities of water. Examples of hydrogel forming compounds include, but are not limited to, polyacrylic acids, sodium carboxymethylcellulose, polyvinyl alcohol, polyvinyl pyrrolidine, gelatin, carrageenan and other polysaccharides, hydroxyethylenemethacrylic acid (HEMA), as well as derivatives thereof, and the like. Hydrogels can be produced that are stable, biodegradable and bioresorbable. Moreover, hydrogel compositions can include subunits that exhibit one or more of these properties.

[0294] Bio-compatible hydrogel compositions whose integrity can be controlled through crosslinking are known and are presently preferred for use in the methods of the invention. For example, Hubbell *et al.*, U.S. Patent Nos. 5,410,016, which issued on April 25, 1995 and 5,529,914, which issued on June 25, 1996, disclose water-soluble systems, which are crosslinked block copolymers having a water-soluble central block segment sandwiched between two hydrolytically labile extensions. Such copolymers are further end-capped with photopolymerizable acrylate functionalities. When crosslinked, these systems become hydrogels. The water soluble central block of such copolymers can include poly(ethylene glycol); whereas, the hydrolytically labile extensions can be a poly(α-hydroxy acid), such as polyglycolic acid or polylactic acid. *See*, Sawhney *et al.*, *Macromolecules* **26**: 581-587 (1993).

[0295] In another preferred embodiment, the gel is a thermoreversible gel. Thermoreversible gels including components, such as pluronics, collagen, gelatin, hyalouronic acid, polysaccharides, polyurethane hydrogel, polyurethane-urea hydrogel and combinations thereof are presently preferred.

[0296] In yet another exemplary embodiment, the conjugate of the invention includes a component of a liposome. Liposomes can be prepared according to methods known to those skilled in the art, for example, as described in Eppstein *et al.*, U.S. Patent No. 4,522,811. For example, liposome formulations may be prepared by dissolving appropriate lipid(s) (such as stearoyl phosphatidyl ethanolamine, stearoyl phosphatidyl choline, arachadoyl phosphatidyl choline, and cholesterol) in an inorganic solvent that is then evaporated, leaving behind a thin film of dried lipid on the surface of the container. An aqueous solution of the active compound or its pharmaceutically acceptable salt is then introduced into the container. The container is then swirled by hand to free lipid material from the sides of the container and to disperse lipid aggregates, thereby forming the liposomal suspension.

[0297] The above-recited microparticles and methods of preparing the microparticles are offered by way of example and they are not intended to define the scope of microparticles of use in the present invention. It will be apparent to those of skill in the art that an array of microparticles, fabricated by different methods, is of use in the present invention.

[0298] The structural formats discussed above in the context of the water-soluble polymers, both straight-chain and branched are generally applicable with respect to the water-insoluble polymers as well. Thus, for example, the cysteine, serine, dilysine, and trilysine

branching cores can be functionalized with two water-insoluble polymer moieties. The methods used to produce these species are generally closely analogous to those used to produce the water-soluble polymers.

Biomolecules

[0299] In another preferred embodiment, the modified sugar bears a biomolecule. In still further preferred embodiments, the biomolecule is a functional protein, enzyme, antigen, antibody, peptide, nucleic acid (*e.g.*, single nucleotides or nucleosides, oligonucleotides, polynucleotides and single- and higher-stranded nucleic acids), lectin, receptor or a combination thereof.

[0300] Preferred biomolecules are essentially non-fluorescent, or emit such a minimal amount of fluorescence that they are inappropriate for use as a fluorescent marker in an assay. Moreover, it is generally preferred to use biomolecules that are not sugars. An exception to this preference is the use of an otherwise naturally occurring sugar that is modified by covalent attachment of another entity (*e.g.*, PEG, biomolecule, therapeutic moiety, diagnostic moiety, *etc.*). In an exemplary embodiment, a sugar moiety, which is a biomolecule, is conjugated to a linker arm and the sugar-linker arm cassette is subsequently conjugated to a peptide via a method of the invention.

[0301] Biomolecules useful in practicing the present invention can be derived from any source. The biomolecules can be isolated from natural sources or they can be produced by synthetic methods. Peptides can be natural peptides or mutated peptides. Mutations can be effected by chemical mutagenesis, site-directed mutagenesis or other means of inducing mutations known to those of skill in the art. Peptides useful in practicing the instant invention include, for example, enzymes, antigens, antibodies and receptors. Antibodies can be either polyclonal or monoclonal; either intact or fragments. The peptides are optionally the products of a program of directed evolution.

[0302] Both naturally derived and synthetic peptides and nucleic acids are of use in conjunction with the present invention; these molecules can be attached to a sugar residue component or a crosslinking agent by any available reactive group. For example, peptides can be attached through a reactive amine, carboxyl, sulfhydryl, or hydroxyl group. The reactive group can reside at a peptide terminus or at a site internal to the peptide chain. Nucleic acids can be attached through a reactive group on a base (*e.g.*, exocyclic amine) or an available hydroxyl group on a sugar moiety (*e.g.*, 3'- or 5'-hydroxyl). The peptide and

nucleic acid chains can be further derivatized at one or more sites to allow for the attachment of appropriate reactive groups onto the chain. *See,* Chrisey *et al. Nucleic Acids Res.* **24:** 3031-3039 (1996).

[0303] In a further preferred embodiment, the biomolecule is selected to direct the peptide modified by the methods of the invention to a specific tissue, thereby enhancing the delivery of the peptide to that tissue relative to the amount of underivatized peptide that is delivered to the tissue. In a still further preferred embodiment, the amount of derivatized peptide delivered to a specific tissue within a selected time period is enhanced by derivatization by at least about 20%, more preferably, at least about 40%, and more preferably still, at least about 100%. Presently, preferred biomolecules for targeting applications include antibodies, hormones and ligands for cell-surface receptors.

[0304] In still a further exemplary embodiment, there is provided as conjugate with biotin. Thus, for example, a selectively biotinylated peptide is elaborated by the attachment of an avidin or streptavidin moiety bearing one or more modifying groups.

The Methods

[0305] In addition to the conjugates discussed above, the present invention provides methods for preparing these and other conjugates. Thus, in a further aspect, the invention provides a method of forming a covalent conjugate between a selected moiety and a peptide. Additionally, the invention provides methods for targeting conjugates of the invention to a particular tissue or region of the body. Furthermore, the present invention provides a method for preventing, curing, or ameliorating a disease state by administering a conjugate of the invention to a subject at risk of developing the disease or a subject that has the disease.

[0306] In exemplary embodiments, the conjugate is formed between a water-soluble polymer, a therapeutic moiety, targeting moiety or a biomolecule, and a glycosylated or non-glycosylated peptide. The polymer, therapeutic moiety or biomolecule is conjugated to the peptide via an intact glycosyl linking group, which is interposed between, and covalently linked to both the peptide and the modifying group (*e.g.*, water-soluble polymer).

[0307] In an exemplary embodiment, the conjugate is formed through a chemical process sometimes referred to as chemoPEGylation. Further discussion of the synthesis chemoPEGylated peptide conjugates is provided in PCT/US02/3226, filed October 9, 2002 and U.S. Pat. App. No. 10/287,994, filed November 5, 2002, each of which are herein incorporated by reference in their entirety.

[0308] The method includes contacting the peptide with a mixture containing a modified sugar and a glycosyltransferase for which the modified sugar is a substrate. The reaction is conducted under conditions sufficient to form a covalent bond between the modified sugar and the peptide. The sugar moiety of the modified sugar is preferably selected from nucleotide sugars, activated sugars, and sugars that are neither nucleotides nor activated.

[0309] The acceptor peptide (glycosylated or non-glycosylated) is typically synthesized *de novo*, or recombinantly expressed in a prokaryotic cell (*e.g.*, bacterial cell, such as *E. coli*) or in a eukaryotic cell such as a mammalian cell (*e.g.*, CHO cells), yeast (*e.g.*, Saccharomyces), insect, or plant cell. The peptide can be either a full-length protein or a fragment. Moreover, the peptide can be a wild type or mutated peptide. In an exemplary embodiment, the peptide includes a mutation that adds one or more consensus glycosylation sites to the peptide sequence.

[0310] The method of the invention also provides for modification of incompletely glycosylated peptides that are produced recombinantly. Many recombinantly produced glycoproteins are incompletely glycosylated, exposing carbohydrate residues that may have undesirable properties, *e.g.*, immunogenicity, recognition by the RES. Employing a modified sugar in a method of the invention, the peptide can be simultaneously further glycosylated and derivatized with, *e.g.*, a water-soluble polymer, therapeutic agent, or the like. The sugar moiety of the modified sugar can be the residue that would properly be conjugated to the acceptor in a fully glycosylated peptide, or another sugar moiety with desirable properties.

[0311] Peptides modified by the methods of the invention can be synthetic or wild-type peptides or they can be mutated peptides, produced by methods known in the art, such as site-directed mutagenesis. Glycosylation of peptides is typically either N-linked or O-linked. An exemplary N-linkage is the attachment of the modified sugar to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of a carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one sugar (*e.g.*, N-aceylgalactosamine, galactose, mannose, GlcNAc, glucose, fucose or xylose) to a the hydroxy side chain of a hydroxyamino acid, preferably serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

[0312] Addition of glycosylation sites to a peptide or other structure is conveniently accomplished by altering the amino acid sequence such that it contains one or more glycosylation sites. The addition may also be made by the incorporation of one or more species presenting an –OH group, preferably serine or threonine residues, within the sequence of the peptide (for O-linked glycosylation sites). The addition may be made by mutation or by full chemical synthesis of the peptide. The peptide amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the peptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) are preferably made using methods known in the art.

[0313] In an exemplary embodiment, the glycosylation site is added by shuffling polynucleotides. Polynucleotides encoding a candidate peptide can be modulated with DNA shuffling protocols. DNA shuffling is a process of recursive recombination and mutation, performed by random fragmentation of a pool of related genes, followed by reassembly of the fragments by a polymerase chain reaction-like process. *See, e.g.*, Stemmer, *Proc. Natl. Acad. Sci. USA* 91:10747-10751 (1994); Stemmer, *Nature* 370:389-391 (1994); and U.S. Patent Nos. 5,605,793, 5,837,458, 5,830,721 and 5,811,238.

[0314] The present invention also provides means of adding (or removing) one or more selected glycosyl residues to a peptide, after which a modified sugar is conjugated to at least one of the selected glycosyl residues of the peptide. The present embodiment is useful, for example, when it is desired to conjugate the modified sugar to a selected glycosyl residue that is either not present on a peptide or is not present in a desired amount. Thus, prior to coupling a modified sugar to a peptide, the selected glycosyl residue is conjugated to the peptide by enzymatic or chemical coupling. In another embodiment, the glycosylation pattern of a glycopeptide is altered prior to the conjugation of the modified sugar by the removal of a carbohydrate residue from the glycopeptide. *See*, for example WO 98/31826.

[0315] Addition or removal of any carbohydrate moieties present on the glycopeptide is accomplished either chemically or enzymatically. Chemical deglycosylation is preferably brought about by exposure of the polypeptide variant to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the peptide intact. Chemical deglycosylation is described by Hakimuddin *et al., Arch. Biochem. Biophys.* **259**: 52 (1987) and by Edge *et al., Anal. Biochem.* **118**: 131 (1981). Enzymatic cleavage of carbohydrate moieties on

polypeptide variants can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al., Meth. Enzymol.* **138**: 350 (1987).

[0316] Chemical addition of glycosyl moieties is carried out by any art-recognized method. Enzymatic addition of sugar moieties is preferably achieved using a modification of the methods set forth herein, substituting native glycosyl units for the modified sugars used in the invention. Other methods of adding sugar moieties are disclosed in U.S. Patent Nos. 5,876,980, 6,030,815, 5,728,554, and 5,922,577.

[0317] Exemplary attachment points for selected glycosyl residue include, but are not limited to: (a) consensus sites for N-linked glycosylation and O-linked glycosylation; (b) terminal glycosyl moieties that are acceptors for a glycosyltransferase; (c) arginine, asparagine and histidine; (d) free carboxyl groups; (e) free sulfhydryl groups such as those of cysteine; (f) free hydroxyl groups such as those of serine, threonine, or hydroxyproline; (g) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan; or (h) the amide group of glutamine. Exemplary methods of use in the present invention are described in WO 87/05330 published Sep. 11, 1987, and in Aplin and Wriston, CRC CRIT. REV. BIOCHEM., pp. 259-306 (1981).

[0318] In one embodiment, the invention provides a method for linking FGF-21 and one or more peptide through a linking group. The linking group is of any useful structure and may be selected from straight-chain and branched chain structures. Preferably, each terminus of the linker, which is attached to a peptide, includes a modified sugar (*i.e.*, a nascent intact glycosyl linking group).

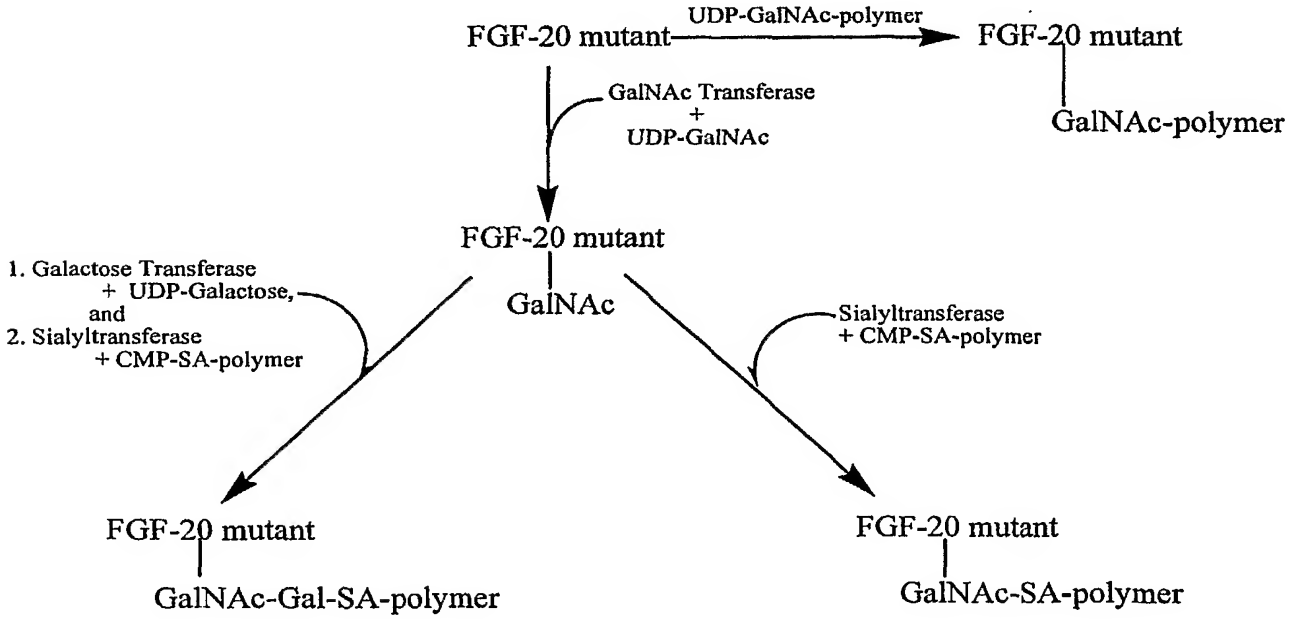
[0319] In an exemplary method of the invention, two peptides are linked together via a linker moiety that includes a PEG linker. The construct conforms to the general structure set forth in the cartoon above. As described herein, the construct of the invention includes two intact glycosyl linking groups (*i.e.*, $s + t = 1$). The focus on a PEG linker that includes two glycosyl groups is for purposes of clarity and should not be interpreted as limiting the identity of linker arms of use in this embodiment of the invention.

[0320] Thus, a PEG moiety is functionalized at a first terminus with a first glycosyl unit and at a second terminus with a second glycosyl unit. The first and second glycosyl units are preferably substrates for different transferases, allowing orthogonal attachment of the first and second peptides to the first and second glycosylunits, respectively. In practice, the $(glycosyl)^1$-PEG-$(glycosyl)^2$ linker is contacted with the first peptide and a first transferase

for which the first glycosyl unit is a substrate, thereby forming (peptide)^1-(glycosyl)^1-PEG-(glycosyl)^2. Glycosyltransferase and/or unreacted peptide is then optionally removed from the reaction mixture. The second peptide and a second transferase for which the second glycosyl unit is a substrate are added to the (peptide)^1-(glycosyl)^1-PEG-(glycosyl)^2 conjugate, forming (peptide)^1-(glycosyl)^1-PEG-(glycosyl)^2-(peptide)^2 . Those of skill in the art will appreciate that the method outlined above is also applicable to forming conjugates between more than two peptides by, for example, the use of a branched PEG, dendrimer, poly(amino acid), polsaccharide or the like.

[0321] Another exemplary embodiment is set forth in Scheme 3. Scheme 3 shows a method of preparing a conjugate comprising a polymer. The polymer increases the circulatory half-life of the FGF protein.

Scheme 3



in which SA is sialic acid, and polymer is PEG, mPEG, poly sialic acid, a water soluble or water insoluble polymer. Though the method is exemplified by reference to FGF-20 and FGF-21, those of skill will appreciate it is equally applicable to other FGF peptides, *e.g.*, FGF-9 and FGF-18.

[0322] The use of reactive derivatives of PEG (or other linkers) to attach one or more peptide moieties to the linker is within the scope of the present invention. The invention is not limited by the identity of the reactive PEG analogue. Many activated derivatives of poly(ethyleneglycol) are available commercially and in the literature. It is well within the abilities of one of skill to choose, and synthesize if necessary, an appropriate activated PEG derivative with which to prepare a substrate useful in the present invention. *See*, Abuchowski *et al. Cancer Biochem. Biophys.*, **7**: 175-186 (1984); Abuchowski *et al.*, *J. Biol. Chem.*, **252**: 3582-3586 (1977); Jackson *et al.*, *Anal. Biochem.*, **165**: 114-127 (1987); Koide *et al.*, *Biochem Biophys. Res. Commun.*, **111**: 659-667 (1983)), tresylate (Nilsson *et al.*, *Methods Enzymol.*, **104**: 56-69 (1984); Delgado *et al.*, *Biotechnol. Appl. Biochem.*, **12**: 119-128 (1990)); N-hydroxysuccinimide derived active esters (Buckmann *et al.*, *Makromol. Chem.*, **182**: 1379-1384 (1981); Joppich *et al.*, *Makromol. Chem.*, **180**: 1381-1384 (1979); Abuchowski *et al.*, *Cancer Biochem. Biophys.*, 7: 175-186 (1984); Katre*et al. Proc. Natl. Acad. Sci. U.S.A.*, **84**: 1487-1491 (1987); Kitamura *et al.*, *Cancer Res.*, **51**: 4310-4315 (1991); Boccu *et al.*, *Z. Naturforsch.*, **38C**: 94-99 (1983), carbonates (Zalipsky *et al.*, POLY(ETHYLENE GLYCOL) CHEMISTRY: BIOTECHNICAL AND BIOMEDICAL APPLICATIONS, Harris, Ed., Plenum Press, New York, 1992, pp. 347-370; Zalipsky *et al.*, *Biotechnol. Appl. Biochem.*, **15**: 100-114 (1992); Veronese *et al.*, *Appl. Biochem. Biotech.*, **11**: 141-152 (1985)), imidazolyl formates (Beauchamp *et al.*, *Anal. Biochem.*, **131**: 25-33 (1983); Berger *et al.*, *Blood*, 71: 1641-1647 (1988)), 4-dithiopyridines (Woghiren *et al.*, *Bioconjugate Chem.*, **4**: 314-318 (1993)), isocyanates (Byun *et al.*, *ASAIO Journal*, M649-M-653 (1992)) and epoxides (U.S. Pat. No. 4,806,595, issued to Noishiki *et al.*, (1989). Other linking groups include the urethane linkage between amino groups and activated PEG. *See*, Veronese, *et al.*, *Appl. Biochem. Biotechnol.*, **11**: 141-152 (1985).

Preparation of Modified Sugars

[0323] In general, the sugar moiety and the modifying group are linked together through the use of reactive groups, which are typically transformed by the linking process into a new organic functional group or species that is unreactive under physiologically relevant conditions. The sugar reactive functional group(s), is located at any position on the sugar moiety. Reactive groups and classes of reactions useful in practicing the present invention are generally those that are well known in the art of bioconjugate chemistry. Currently favored classes of reactions available with reactive sugar moieties are those, which proceed under relatively mild conditions. These include, but are not limited to nucleophilic

substitutions (*e.g.*, reactions of amines and alcohols with acyl halides, active esters), electrophilic substitutions (*e.g.*, enamine reactions) and additions to carbon-carbon and carbon-heteroatom multiple bonds (*e.g.*, Michael reaction, Diels-Alder addition). These and other useful reactions are discussed in, for example, March, ADVANCED ORGANIC CHEMISTRY, 3rd Ed., John Wiley & Sons, New York, 1985; Hermanson, BIOCONJUGATE TECHNIQUES, Academic Press, San Diego, 1996; and Feeney *et al.*, MODIFICATION OF PROTEINS; Advances in Chemistry Series, Vol. 198, American Chemical Society, Washington, D.C., 1982.

[0324] Useful reactive functional groups pendent from a sugar nucleus or modifying group include, but are not limited to:

(a) carboxyl groups and various derivatives thereof including, but not limited to, N-hydroxysuccinimide esters, N-hydroxybenztriazole esters, acid halides, acyl imidazoles, thioesters, p-nitrophenyl esters, alkyl, alkenyl, alkynyl and aromatic esters;

(b) hydroxyl groups, which can be converted to, *e.g.*, esters, ethers, aldehydes, *etc.*

(c) haloalkyl groups, wherein the halide can be later displaced with a nucleophilic group such as, for example, an amine, a carboxylate anion, thiol anion, carbanion, or an alkoxide ion, thereby resulting in the covalent attachment of a new group at the functional group of the halogen atom;

(d) dienophile groups, which are capable of participating in Diels-Alder reactions such as, for example, maleimido groups;

(e) aldehyde or ketone groups, such that subsequent derivatization is possible via formation of carbonyl derivatives such as, for example, imines, hydrazones, semicarbazones or oximes, or via such mechanisms as Grignard addition or alkyllithium addition;

(f) sulfonyl halide groups for subsequent reaction with amines, for example, to form sulfonamides;

(g) thiol groups, which can be, for example, converted to disulfides or reacted with acyl halides;

(h) amine or sulfhydryl groups, which can be, for example, acylated, alkylated or oxidized;

(i) alkenes, which can undergo, for example, cycloadditions, acylation, Michael addition, *etc*; and

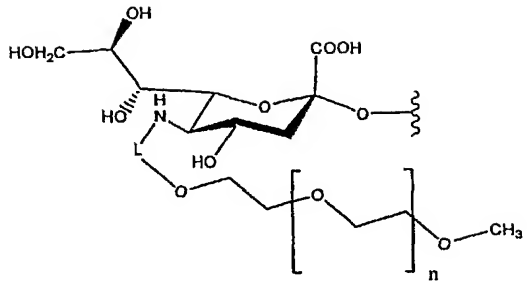
(j) epoxides, which can react with, for example, amines and hydroxyl compounds.

[0325] The reactive functional groups can be chosen such that they do not participate in, or interfere with, the reactions necessary to assemble the reactive sugar nucleus or modifying group. Alternatively, a reactive functional group can be protected from participating in the reaction by the presence of a protecting group. Those of skill in the art understand how to protect a particular functional group such that it does not interfere with a chosen set of reaction conditions. For examples of useful protecting groups, *see,* for example, Greene *et al.*, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS, John Wiley & Sons, New York, 1991.

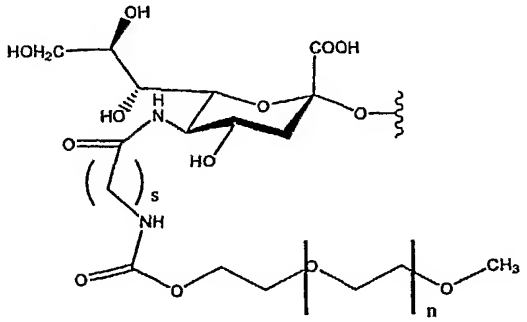
[0326] In the discussion that follows, a number of specific examples of modified sugars that are useful in practicing the present invention are set forth. In the exemplary embodiments, a sialic acid derivative is utilized as the sugar nucleus to which the modifying group is attached. The focus of the discussion on sialic acid derivatives is for clarity of illustration only and should not be construed to limit the scope of the invention. Those of skill in the art will appreciate that a variety of other sugar moieties can be activated and derivatized in a manner analogous to that set forth using sialic acid as an example. For example, numerous methods are available for modifying galactose, glucose, N-acetylgalactosamine and fucose to name a few sugar substrates, which are readily modified by art recognized methods. *See*, for example, Elhalabi *et al.*, *Curr. Med. Chem.* **6**: 93 (1999); and Schafer *et al.*, *J. Org. Chem.* **65**: 24 (2000)).

[0327] In an exemplary embodiment, the peptide that is modified by a method of the invention is a glycopeptide that is produced in prokaryotic cells *(e.g., E.coli)*, eukaryotic cells including yeast and mammalian cells (*e.g.*, CHO cells), or in a transgenic animal and thus, contains N- and/or O-linked oligosaccharide chains, which are incompletely sialylated. The oligosaccharide chains of the glycopeptide lacking a sialic acid and containing a terminal galactose residue can be PEG-ylated, PPG-ylated or otherwise modified with a modified sialic acid.

[0328] Exemplary PEG-sialic acid derivative include:



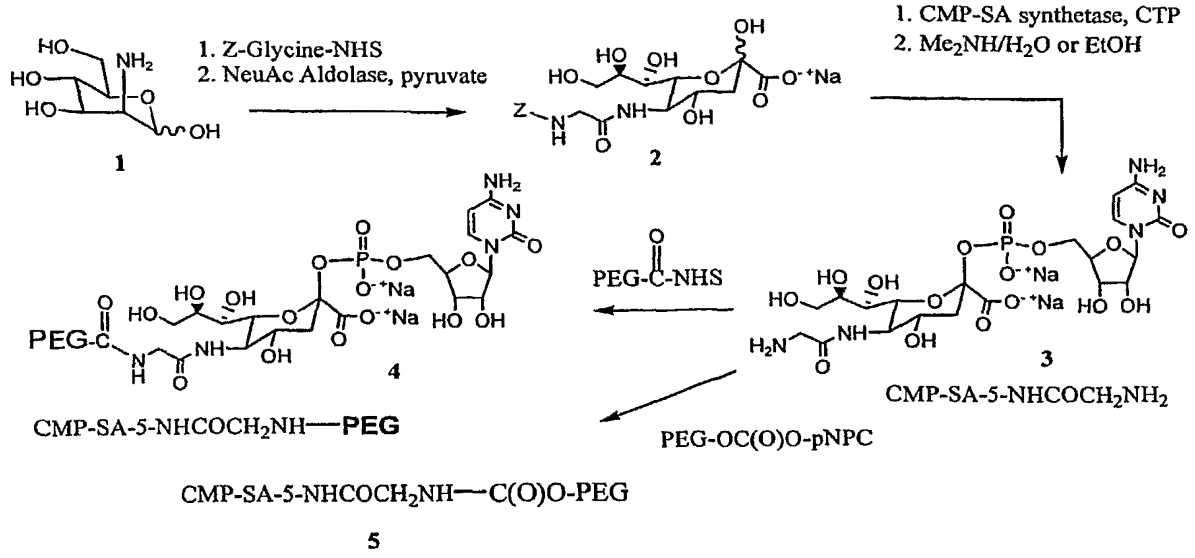
in which L is a substituted or unsubstituted alkyl or substituted or unsubstituted heteroalkyl linker moiety joining the sialic acid moiety and the PEG moiety, and "n" is 1 or greater; and



in which the index "s" represents an integer from 0 to 20, and "n" is 1 or greater.

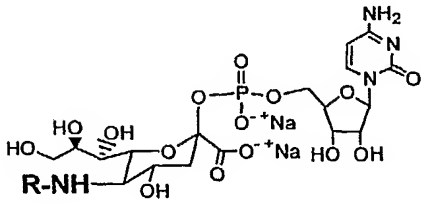
[0329] In Scheme 4, the amino glycoside **1**, is treated with the active ester of a protected amino acid (*e.g.*, glycine) derivative, converting the sugar amine residue into the corresponding protected amino acid amide adduct. The adduct is treated with an aldolase to form α-hydroxy carboxylate **2**. Compound **2** is converted to the corresponding CMP derivative by the action of CMP-SA synthetase, followed by catalytic hydrogenation of the CMP derivative to produce compound **3**. The amine introduced via formation of the glycine adduct is utilized as a locus of PEG attachment by reacting compound **3** with an activated PEG or PPG derivative (*e.g.*, PEG-C(O)NHS, PEG-OC(O)O-p-nitrophenyl), producing species such as **4** or **5**, respectively.

Scheme 4

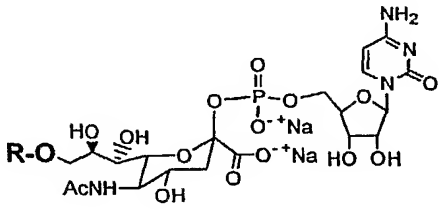


[0330] Table 1 sets forth representative examples of sugar monophosphates that are derivatized with a modifying group, such as a PEG or PPG moiety. Fibroblast Growth Factor peptides can be modified by the method of Scheme 1. Other derivatives are prepared by art-recognized methods. *See*, for example, Keppler *et al.*, *Glycobiology* **11**: 11R (2001); and Charter *et al.*, *Glycobiology* **10**: 1049 (2000)). Other amine reactive PEG and PPG analogues are commercially available, or they can be prepared by methods readily accessible to those of skill in the art.

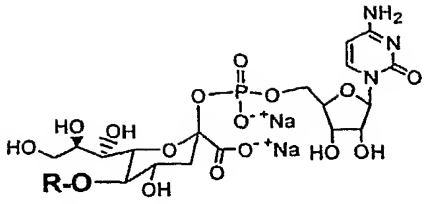
Table 1



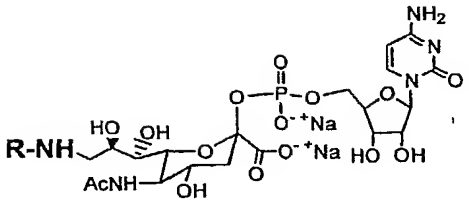
CMP-SA-5-NH-R



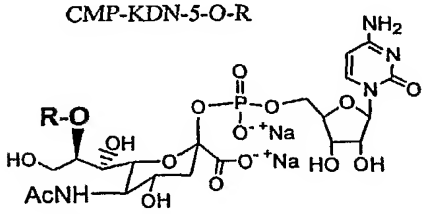
CMP-NeuAc-9-O-R



CMP-KDN-5-O-R



CMP-NeuAc-9-NH-R



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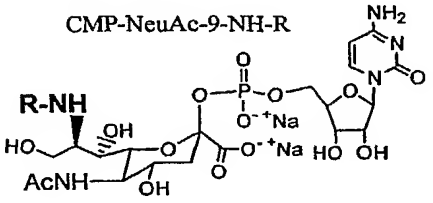

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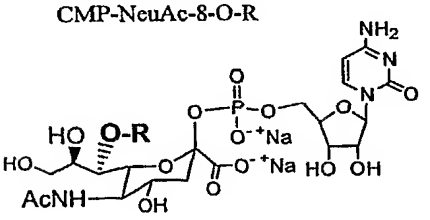

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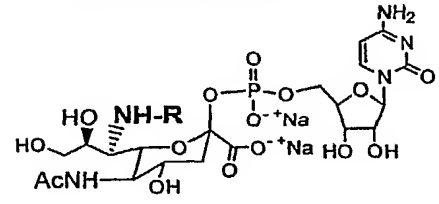

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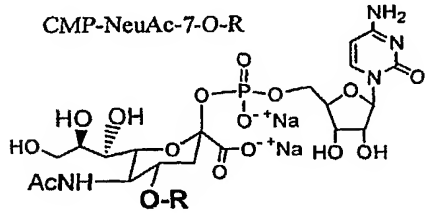
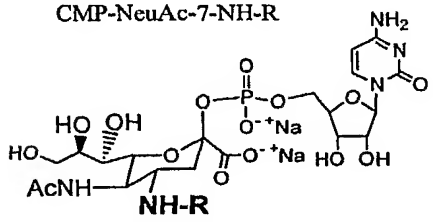


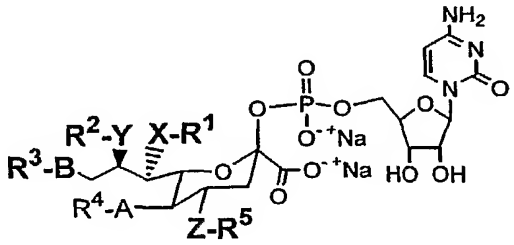
CMP-NeuAc-4-O-R



CMP-NeuAc-4-NH-R

wherein R represents a modifying group, *e.g.*, linear or branched PEG or $-L^x-R^x$ in which L^x is a linker selected from a bond (zero-order), substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl, and R^x is the modifying group.

[0331] The modified sugar phosphates of use in practicing the present invention can be substituted in other positions as well as those set forth above. Presently preferred substitutions of sialic acid are set forth in Formula I:



(I)

in which X is a linking group, which is preferably selected from –O-, -N(H)-, -S, CH_2-, and -$N(R)_2$, in which each R is a member independently selected from R^1-R^5. The symbols Y, Z,

A and B each represent a group that is selected from the group set forth above for the identity of X. X, Y, Z, A and B are each independently selected and, therefore, they can be the same or different. The symbols R^1, R^2, R^3, R^4 and R^5 represent H, a water-soluble polymer, therapeutic moiety, biomolecule or other moiety. Alternatively, these symbols represent a linker that is bound to a water-soluble polymer, therapeutic moiety, biomolecule or other moiety.

[0332] Exemplary moieties attached to the conjugates disclosed herein include, but are not limited to, PEG derivatives (*e.g.*, alkyl-PEG, acyl-PEG, acyl-alkyl-PEG, alkyl-acyl-PEG carbamoyl-PEG, aryl-PEG), PPG derivatives (*e.g.*, alkyl-PPG, acyl-PPG, acyl-alkyl-PPG, alkyl-acyl-PPG carbamoyl-PPG, aryl-PPG), therapeutic moieties, diagnostic moieties, mannose-6-phosphate, heparin, heparan, SLe_x, mannose, mannose-6-phosphate, Sialyl Lewis X, FGF, VFGF, proteins, chondroitin, keratan, dermatan, albumin, integrins, antennary oligosaccharides, peptides and the like. Methods of conjugating the various modifying groups to a saccharide moiety are readily accessible to those of skill in the art (POLY (ETHYLENE GLYCOL CHEMISTRY : BIOTECHNICAL AND BIOMEDICAL APPLICATIONS, J. Milton Harris, Ed., Plenum Pub. Corp., 1992; POLY (ETHYLENE GLYCOL) CHEMICAL AND BIOLOGICAL APPLICATIONS, J. Milton Harris, Ed., ACS Symposium Series No. 680, American Chemical Society, 1997; Hermanson, BIOCONJUGATE TECHNIQUES, Academic Press, San Diego, 1996; and Dunn *et al.*, Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991).

Cross-linking Groups

[0333] Preparation of the modified sugar for use in the methods of the present invention includes attachment of a modifying group to a sugar residue and forming a stable adduct, which is a substrate for a glycosyltransferase. The sugar and modifying group can be coupled by a zero- or higher-order cross-linking agent. Exemplary bifunctional compounds which can be used for attaching modifying groups to carbohydrate moieties include, but are not limited to, bifunctional poly(ethyleneglycols), polyamides, polyethers, polyesters and the like. General approaches for linking carbohydrates to other molecules are known in the literature. *See,* for example, Lee *et al.*, *Biochemistry* **28**: 1856 (1989); Bhatia *et al.*, *Anal. Biochem.* **178**: 408 (1989); Janda *et al.*, *J. Am. Chem. Soc.* **112**: 8886 (1990) and Bednarski *et al.*, WO 92/18135. In the discussion that follows, the reactive groups are treated as benign on the sugar moiety of the nascent modified sugar. The focus of the discussion is for clarity of

illustration. Those of skill in the art will appreciate that the discussion is relevant to reactive groups on the modifying group as well.

[0334] A variety of reagents are used to modify the components of the modified sugar with intramolecular chemical crosslinks (for reviews of crosslinking reagents and crosslinking procedures see: Wold, F., *Meth. Enzymol.* **25**: 623-651, 1972; Weetall, H. H., and Cooney, D. A., In: ENZYMES AS DRUGS. (Holcenberg, and Roberts, eds.) pp. 395-442, Wiley, New York, 1981; Ji, T. H., *Meth. Enzymol.* **91**: 580-609, 1983; Mattson *et al.*, *Mol. Biol. Rep.* **17**: 167-183, 1993, all of which are incorporated herein by reference). Preferred crosslinking reagents are derived from various zero-length, homo-bifunctional, and hetero-bifunctional crosslinking reagents. Zero-length crosslinking reagents include direct conjugation of two intrinsic chemical groups with no introduction of extrinsic material. Agents that catalyze formation of a disulfide bond belong to this category. Another example is reagents that induce condensation of a carboxyl and a primary amino group to form an amide bond such as carbodiimides, ethylchloroformate, Woodward's reagent K (2-ethyl-5-phenylisoxazolium-3'-sulfonate), and carbonyldiimidazole. In addition to these chemical reagents, the enzyme transglutaminase (glutamyl-peptide γ-glutamyltransferase; EC 2.3.2.13) may be used as zero-length crosslinking reagent. This enzyme catalyzes acyl transfer reactions at carboxamide groups of protein-bound glutaminyl residues, usually with a primary amino group as substrate. Preferred homo- and hetero-bifunctional reagents contain two identical or two dissimilar sites, respectively, which may be reactive for amino, sulfhydryl, guanidino, indole, or nonspecific groups.

[0335] In still another embodiment, photoactivatable groups are selected from diazopyruvates. For example, the p-nitrophenyl ester of p-nitrophenyl diazopyruvate reacts with aliphatic amines to give diazopyruvic acid amides that undergo ultraviolet photolysis to form aldehydes. The photolyzed diazopyruvate-modified affinity component will react like formaldehyde or glutaraldehyde forming crosslinks.

Cleavable Linker Groups

[0336] In yet a further embodiment, the linker group is provided with a group that can be cleaved to release the modifying group from the sugar residue. Many cleaveable groups are known in the art. *See,* for example, Jung *et al.*, *Biochem. Biophys. Acta* **761**: 152-162 (1983); Joshi *et al.*, *J. Biol. Chem.* **265**: 14518-14525 (1990); Zarling *et al.*, *J. Immunol.* **124**: 913-920 (1980); Bouizar *et al.*, *Eur. J. Biochem.* **155**: 141-147 (1986); Park *et al.*, *J. Biol. Chem.* **261**:

205-210 (1986); Browning *et al., J. Immunol.* **143**: 1859-1867 (1989). Moreover a broad range of cleavable, bifunctional (both homo- and hetero-bifunctional) linker groups is commercially available from suppliers such as Pierce.

[0337] Exemplary cleaveable moieties can be cleaved using light, heat or reagents such as thiols, hydroxylamine, bases, periodate and the like. Moreover, certain preferred groups are cleaved *in vivo* in response to being endocytized (*e.g.*, cis-aconityl; *see,* Shen *et al., Biochem. Biophys. Res. Commun.* **102**: 1048 (1991)). Preferred cleaveable groups comprise a cleaveable moiety which is a member selected from the group consisting of disulfide, ester, imide, carbonate, nitrobenzyl, phenacyl and benzoin groups.

Conjugation of Modified Sugars to Peptides

[0338] The modified sugars are conjugated to a glycosylated or non-glycosylated peptide using an appropriate enzyme to mediate the conjugation. Preferably, the concentrations of the modified donor sugar(s), enzyme(s) and acceptor peptide(s) are selected such that glycosylation proceeds until the acceptor is consumed. The considerations discussed below, while set forth in the context of a sialyltransferase, are generally applicable to other glycosyltransferase reactions.

[0339] A number of methods of using glycosyltransferases to synthesize desired oligosaccharide structures are known and are generally applicable to the instant invention. Exemplary methods are described, for instance, WO 96/32491, Ito *et al., Pure Appl. Chem.* **65**: 753 (1993), and U.S. Pat. Nos. 5,352,670, 5,374,541, and 5,545,553.

[0340] The present invention is practiced using a single glycosyltransferase or a combination of glycosyltransferases. For example, one can use a combination of a sialyltransferase and a galactosyltransferase. In those embodiments using more than one enzyme, the enzymes and substrates are preferably combined in an initial reaction mixture, or the enzymes and reagents for a second enzymatic reaction are added to the reaction medium once the first enzymatic reaction is complete or nearly complete. By conducting two enzymatic reactions in sequence in a single vessel, overall yields are improved over procedures in which an intermediate species is isolated. Moreover, cleanup and disposal of extra solvents and by-products is reduced.

[0341] In a preferred embodiment, each of the first and second enzyme is a glycosyltransferase. In another preferred embodiment, one enzyme is an endoglycosidase. In an additional preferred embodiment, more than two enzymes are used to assemble the

modified glycoprotein of the invention. The enzymes are used to alter a saccharide structure on the peptide at any point either before or after the addition of the modified sugar to the peptide.

[0342] In another embodiment, the method makes use of one or more exo- or endoglycosidase. The glycosidase is typically a mutant, which is engineered to form glycosyl bonds rather than cleave them. The mutant glycanase typically includes a substitution of an amino acid residue for an active site acidic amino acid residue. For example, when the endoglycanase is endo-H, the substituted active site residues will typically be Asp at position 130, Glu at position 132 or a combination thereof. The amino acids are generally replaced with serine, alanine, asparagine, or glutamine.

[0343] The mutant enzyme catalyzes the reaction, usually by a synthesis step that is analogous to the reverse reaction of the endoglycanase hydrolysis step. In these embodiments, the glycosyl donor molecule (*e.g.*, a desired oligo- or mono-saccharide structure) contains a leaving group and the reaction proceeds with the addition of the donor molecule to a GlcNAc residue on the protein. For example, the leaving group can be a halogen, such as fluoride. In other embodiments, the leaving group is a Asn, or a Asn-peptide moiety. In yet further embodiments, the GlcNAc residue on the glycosyl donor molecule is modified. For example, the GlcNAc residue may comprise a 1,2 oxazoline moiety.

[0344] In a preferred embodiment, each of the enzymes utilized to produce a conjugate of the invention are present in a catalytic amount. The catalytic amount of a particular enzyme varies according to the concentration of that enzyme's substrate as well as to reaction conditions such as temperature, time and pH value. Means for determining the catalytic amount for a given enzyme under preselected substrate concentrations and reaction conditions are well known to those of skill in the art.

[0345] The temperature at which an above process is carried out can range from just above freezing to the temperature at which the most sensitive enzyme denatures. Preferred temperature ranges are about 0 °C to about 55 °C, and more preferably about 20 ° C to about 30 °C. In another exemplary embodiment, one or more components of the present method are conducted at an elevated temperature using a thermophilic enzyme.

[0346] The reaction mixture is maintained for a period of time sufficient for the acceptor to be glycosylated, thereby forming the desired conjugate. Some of the conjugate can often be

detected after a few hours, with recoverable amounts usually being obtained within 24 hours or less. Those of skill in the art understand that the rate of reaction is dependent on a number of variable factors (*e.g*, enzyme concentration, donor concentration, acceptor concentration, temperature, solvent volume), which are optimized for a selected system.

[0347] The present invention also provides for the industrial-scale production of modified peptides. As used herein, an industrial scale generally produces at least about 250 mg, preferably at least about 500 mg, and more preferably at least about 1 gram of finished, purified conjugate, preferably after a single reaction cycle, *i.e.*, the conjugate is not a combination the reaction products from identical, consecutively iterated synthesis cycles.

[0348] In the discussion that follows, the invention is exemplified by the conjugation of modified sialic acid moieties to a glycosylated peptide. The exemplary modified sialic acid is labeled with m-PEG. The focus of the following discussion on the use of PEG-modified sialic acid and glycosylated peptides is for clarity of illustration and is not intended to imply that the invention is limited to the conjugation of these two partners. One of skill understands that the discussion is generally applicable to the additions of modified glycosyl moieties other than sialic acid. Moreover, the discussion is equally applicable to the modification of a glycosyl unit with agents other than m-PEG including other water-soluble polymers, therapeutic moieties, and biomolecules.

[0349] An enzymatic approach can be used for the selective introduction of PEGylated or PPGylated carbohydrates onto a peptide or glycopeptide. The method utilizes modified sugars containing PEG, PPG, or a masked reactive functional group, and is combined with the appropriate glycosyltransferase or glycosynthase. By selecting the glycosyltransferase that will make the desired carbohydrate linkage and utilizing the modified sugar as the donor substrate, the PEG or PPG can be introduced directly onto the peptide backbone, onto existing sugar residues of a glycopeptide or onto sugar residues that have been added to a peptide.

[0350] An acceptor for the sialyltransferase is present on the peptide to be modified by the methods of the present invention either as a naturally occurring structure or one placed there recombinantly, enzymatically or chemically. Suitable acceptors, include, for example, galactosyl acceptors such as Galβ1,4GlcNAc, Galβ1,4GalNAc, Galβ1,3GalNAc, lacto-N-tetraose, Galβ1,3GlcNAc, GalNAc, Galβ1,3GalNAc, Galβ1,6GlcNAc, Galβ1,4Glc (lactose),

and other acceptors known to those of skill in the art (*see, e.g.*, Paulson *et al., J. Biol. Chem.* **253**: 5617-5624 (1978)).

[0351] In one embodiment, an acceptor for the sialyltransferase is present on the glycopeptide to be modified upon *in vivo* synthesis of the glycopeptide. Such glycopeptides can be sialylated using the claimed methods without prior modification of the glycosylation pattern of the glycopeptide. Alternatively, the methods of the invention can be used to sialylate a peptide that does not include a suitable acceptor; one first modifies the peptide to include an acceptor by methods known to those of skill in the art. In an exemplary embodiment, a GalNAc residue is added by the action of a GalNAc transferase.

[0352] In an exemplary embodiment, the galactosyl acceptor is assembled by attaching a galactose residue to an appropriate acceptor linked to the peptide, *e.g.*, a GalNAc. The method includes incubating the peptide to be modified with a reaction mixture that contains a suitable amount of a galactosyltransferase (*e.g.*, Galβ1,3 or Galβ1,4), and a suitable galactosyl donor (*e.g.*, UDP-galactose). The reaction is allowed to proceed substantially to completion or, alternatively, the reaction is terminated when a preselected amount of the galactose residue is added. Other methods of assembling a selected saccharide acceptor will be apparent to those of skill in the art.

[0353] In yet another embodiment, glycopeptide-linked oligosaccharides are first "trimmed," either in whole or in part, to expose either an acceptor for the sialyltransferase or a moiety to which one or more appropriate residues can be added to obtain a suitable acceptor. Enzymes such as glycosyltransferases and endoglycosidases (*see*, for example U.S. Patent No. 5,716,812) are useful for the attaching and trimming reactions.

[0354] In the discussion that follows, the method of the invention is exemplified by the use of modified sugars having a water-soluble polymer attached thereto. The focus of the discussion is for clarity of illustration. Those of skill will appreciate that the discussion is equally relevant to those embodiments in which the modified sugar bears a therapeutic moiety, biomolecule or the like.

[0355] In an exemplary embodiment, an O-linked carbohydrate residue is "trimmed" prior to the addition of the modified sugar. For example a GalNAc-Gal residue is trimmed back to GalNAc. A modified sugar bearing a water-soluble polymer is conjugated to one or more of the sugar residues exposed by the "trimming." In one example, a glycopeptide is "trimmed" and a water-soluble polymer is added to the resulting O-side chain amino acid or

glycopeptide glycan via a saccharyl moiety, *e.g.*, Sia, Gal, or GalNAc moiety conjugated to the water-soluble polymer. The modified saccharyl moiety is attached to an acceptor site on the "trimmed" glycopeptide. Alternatively, an unmodified saccharyl moiety, *e.g.*, Gal can be added the terminus of the O-linked glycan.

[0356] In another exemplary embodiment, a water-soluble polymer is added to a GalNAc residue via a modified sugar having a galactose residue. Alternatively, an unmodified Gal can be added to the terminal GalNAc residue.

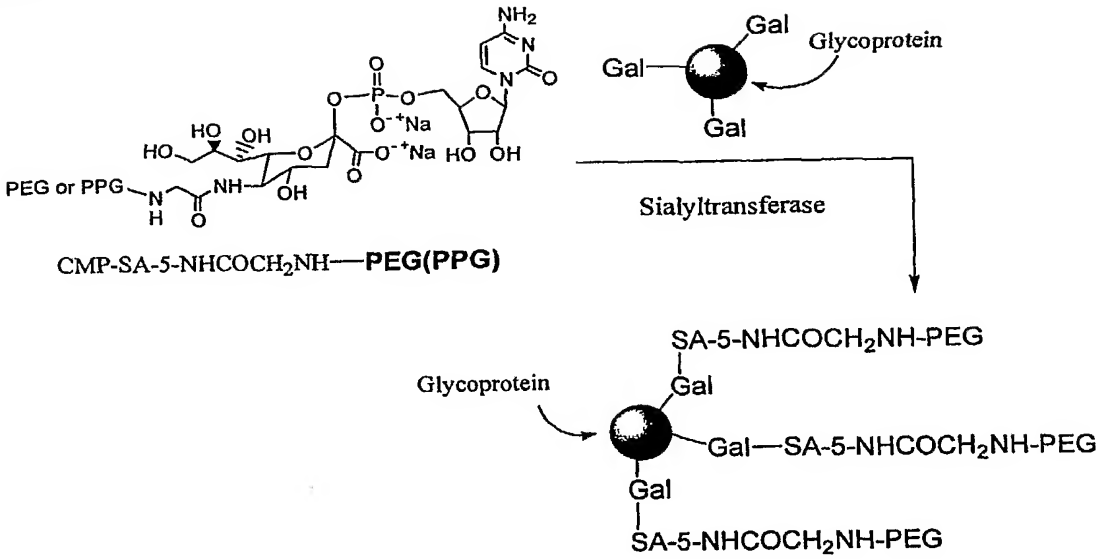
[0357] In yet a further example, a water-soluble polymer is added onto a Gal residue using a modified sialic acid.

[0358] In another exemplary embodiment, an O-linked glycosyl residue is "trimmed back" to the GalNAc attached to the amino acid. In one example, a water-soluble polymer is added via a Gal modified with the polymer. Alternatively, an unmodified Gal is added to the GalNAc, followed by a Gal with an attached water-soluble polymer. In yet another embodiment, one or more unmodified Gal residue is added to the GalNAc, followed by a sialic acid moiety modified with a water-soluble polymer.

[0359] Using the methods of the invention, it is possible to "trim back" and build up a carbohydrate residue of substantially any desired structure. The modified sugar can be added to the termini of the carbohydrate moiety as set forth above, or it can be intermediate between the peptide core and the terminus of the carbohydrate.

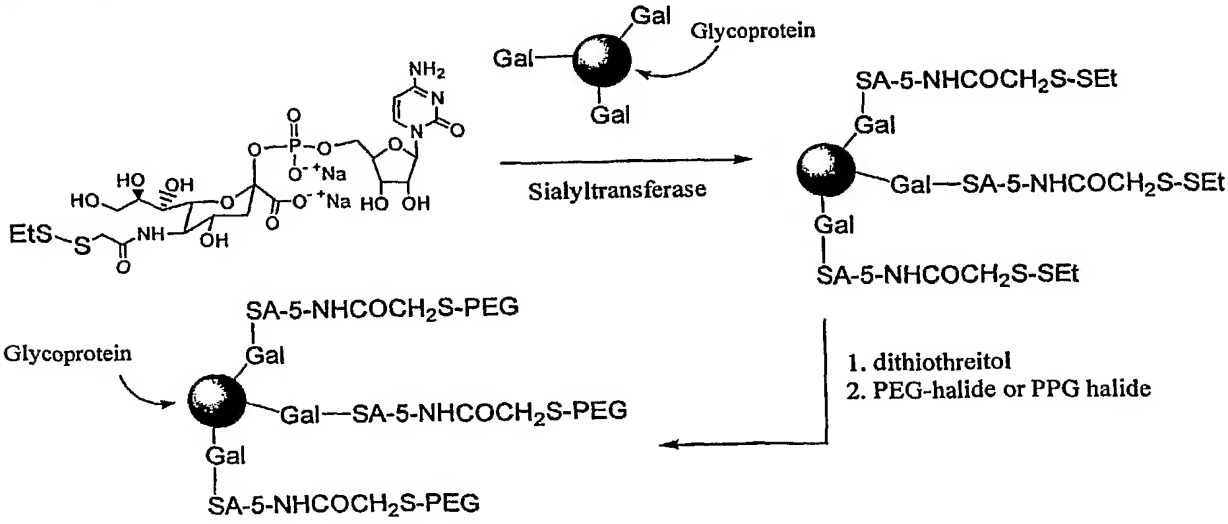
[0360] In an exemplary embodiment, the water-soluble polymer is added to a terminal Gal residue using a polymer modified sialic acid. An appropriate sialyltransferase is used to add a modified sialic acid. The approach is summarized in Scheme 5.

Scheme 5



[0361] In yet a further approach, summarized in Scheme 6, a masked reactive functionality is present on the sialic acid. The masked reactive group is preferably unaffected by the conditions used to attach the modified sialic acid to the peptide. After the covalent attachment of the modified sialic acid to the peptide, the mask is removed and the peptide is conjugated with an agent such as PEG, PPG, a therapeutic moiety, biomolecule or other agent. The agent is conjugated to the peptide in a specific manner by its reaction with the unmasked reactive group on the modified sugar residue.

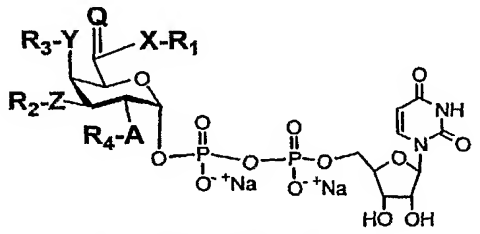
Scheme 6



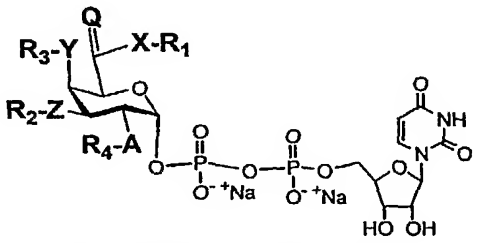
[0362] Any modified sugar can be used with its appropriate glycosyltransferase, depending on the terminal sugars of the oligosaccharide side chains of the glycopeptide (Table 2). As

discussed above, the terminal sugar of the glycopeptide required for introduction of the PEG-ylated or PPG-ylated structure can be introduced naturally during expression or it can be produced post expression using the appropriate glycosidase(s), glycosyltransferase(s) or mix of glycosidase(s) and glycosyltransferase(s).

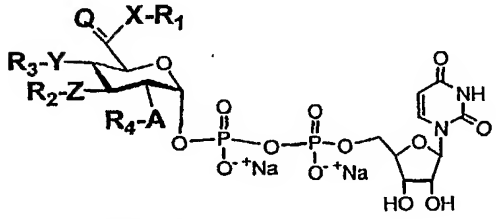
Table 2



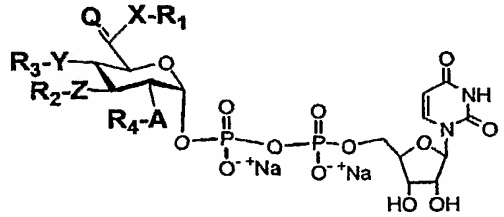
UDP-galactose-derivatives



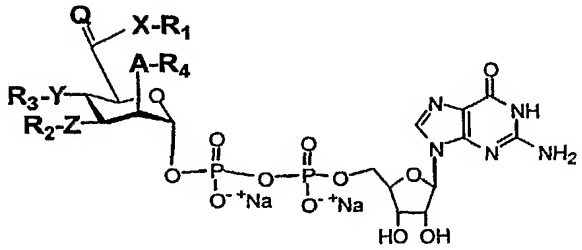
UDP-galactosamine-derivatives
(when A = NH, R_4 may be acetyl)



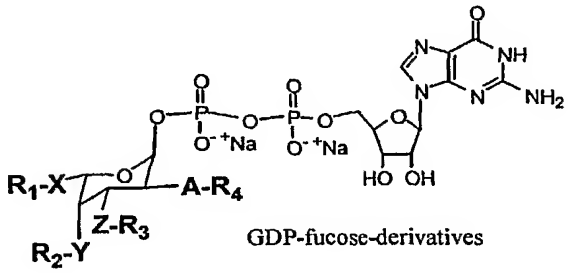
UDP-Glucose-derivatives



UDP-Glucosamine-derivatives
(when A = NH, R_4 may be acetyl)



GDP-Mannose-derivatives



GDP-fucose-derivatives

X = O, NH, S, CH_2, N-(R_1-5)2. Y = X; Z = X; A = X; B = X. Q = H_2, O, S, NH, N-R. R, R_1-4 = H, Linker-M, M. M = Ligand of interest	Ligand of interest = acyl-PEG, acyl-PPG, alkyl-PEG, acyl-alkyl-PEG, acyl-alkyl-PEG, carbamoyl-PEG, carbamoyl-PPG, PEG, PPG, acyl-aryl-PEG, acyl-aryl-PPG, aryl-PEG, aryl-PPG, Mannose-6-phosphate, heparin, heparan, SLex, Mannose, FGF, VFGF, protein, chondroitin, keratan, dermatan, albumin, integrins, peptides, etc.

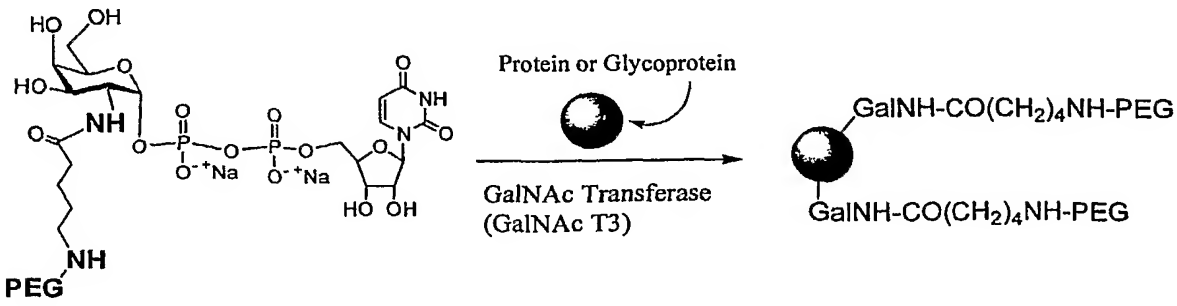
[0363] In a further exemplary embodiment, UDP-galactose-PEG is reacted with bovine milk β1,4-galactosyltransferase, thereby transferring the modified galactose to the appropriate terminal N-acetylglucosamine structure. The terminal GlcNAc residues on the glycopeptide may be produced during expression, as may occur in such expression systems as mammalian,

insect, plant or fungus, but also can be produced by treating the glycopeptide with a sialidase and/or glycosidase and/or glycosyltransferase, as required.

[0364] In another exemplary embodiment, a GlcNAc transferase, such as GNT1-5, is utilized to transfer PEGylated-GlcN to a terminal mannose residue on a glycopeptide. In a still further exemplary embodiment, an the N- and/or O-linked glycan structures are enzymatically removed from a glycopeptide to expose an amino acid or a terminal glycosyl residue that is subsequently conjugated with the modified sugar. For example, an endoglycanase is used to remove the N-linked structures of a glycopeptide to expose a terminal GlcNAc as a GlcNAc-linked-Asn on the glycopeptide. UDP-Gal-PEG and the appropriate galactosyltransferase is used to introduce the PEG- or PPG-galactose functionality onto the exposed GlcNAc.

[0365] In an alternative embodiment, the modified sugar is added directly to the peptide backbone using a glycosyltransferase known to transfer sugar residues to the peptide backbone. This exemplary embodiment is set forth in Scheme 7. Exemplary glycosyltransferases useful in practicing the present invention include, but are not limited to, GalNAc transferases (GalNAc T1-20), GlcNAc transferases, fucosyltransferases, glucosyltransferases, xylosyltransferases, mannosyltransferases and the like. Use of this approach allows the direct addition of modified sugars onto peptides that lack any carbohydrates or, alternatively, onto existing glycopeptides. In both cases, the addition of the modified sugar occurs at specific positions on the peptide backbone as defined by the substrate specificity of the glycosyltransferase and not in a random manner as occurs during modification of a protein's peptide backbone using chemical methods. An array of agents can be introduced into proteins or glycopeptides that lack the glycosyltransferase substrate peptide sequence by engineering the appropriate amino acid sequence into the polypeptide chain.

Scheme 7



[0366] In each of the exemplary embodiments set forth above, one or more additional chemical or enzymatic modification steps can be utilized following the conjugation of the modified sugar to the peptide. In an exemplary embodiment, an enzyme (*e.g.*, fucosyltransferase) is used to append a glycosyl unit (*e.g.*, fucose) onto the terminal modified sugar attached to the peptide. In another example, an enzymatic reaction is utilized to "cap" (*e.g.*, sialylate) sites to which the modified sugar failed to conjugate. Alternatively, a chemical reaction is utilized to alter the structure of the conjugated modified sugar. For example, the conjugated modified sugar is reacted with agents that stabilize or destabilize its linkage with the peptide component to which the modified sugar is attached. In another example, a component of the modified sugar is deprotected following its conjugation to the peptide. One of skill will appreciate that there is an array of enzymatic and chemical procedures that are useful in the methods of the invention at a stage after the modified sugar is conjugated to the peptide. Further elaboration of the modified sugar-peptide conjugate is within the scope of the invention.

Enzyme Classes

[0367] Aspects of the present invention make use of enzymes that form a bond between an activated acyl moiety and a heteroatom found on a sugar nucleus. The enzymes useful in practicing the present invention include, but are not limited to, wild-type and mutant proteases, lipases, esterases, acylases, acyltransferases, glycosyltransferases, sufotransferases, glycosidases, and the like. An exemplary mutant is one in which one or more amino acid residues in the active site are altered to provide an enzyme with synthetic activity that is improved relative to the activity in the corresponding wild-type enzyme.

Acyl Transfer

[0368] The discovery that some enzymes are catalytically active in organic solvents has greatly expanded their use as biocatalysts. In this medium these enzymes show a new catalytic behavior. For example lipases catalyse esterification and transesterification reactions in organic media. These properties enable the production of compounds which are difficult to obtain using chemical methods.

Proteases

[0369] A protease is employed in some embodiments of the invention. Proteases are known in the art to catalyze the attachment of amino acids to sugars through esterification. (Davis, (WO 03/014371, published Feb. 20, 2003). In this publication, a vinyl ester amino acid group was reacted with a carbohydrate acyl acceptor in the presence of the serine

protease subtilisin derived from *Bacillus lentus*. Wild-type proteases can be additionally be isolated from *Bacillus amyloliquefaciens*. Mutant proteases can be made according to the teachings of, for example, PCT Publication Nos. WO 95/10615 and WO 91/06637, which are hereby incorporated by reference. Other proteases of use in this invention include serine proteases (such as chymotrypsin, plasmin, and thrombin), cysteine proteases (such as cathepsin B and papain), and aspartic endopeptidases (such as pepsin A, chymosin, cathepsin D, asparagenase).

[0370] In an exemplary embodiment, utilizing a protease, the link between the sugar moiety and the modifying group is an amino acid that is derivatized with the modifying group. The sugar and amino acid are linked through an amide moiety formed by the protease.

Lipases

[0371] A lipase is used in some embodiments of the invention. The use of lipases in the acylation of saccharides has been previously reported. For example, regioselective acylations of alkyl β-D-xylopyranosides using lipase PS in organic solvents was reported by Lopez. (Lopez *et al.*, *J. Org. Chem.*, **59**, 7027-7032 (1994). Another group also utilized lipase PS in order to catalyze the transfer of acetyl groups onto sialic acids in vinyl acetate. (Lo *et al.*, *Bioorg. Med. Chem. Lett.*, **9**, 709-712 (1999)). Regioselective disaccharide acylation in *tert*-butyl alcohol catalyzed by *Candida antarctica* lipase has also been reported. (Woudenberg van-Oosterom *et al.*, *Biotechnol. Bioeng.*, **49**, 328-333 (1996)). Immobilized versions of the *Candida antarctica* lipase have also been used to acylate hydroxypropyl cellulose in tert-butanol. (Sereti *et al.*, *Biotechnol Bioeng.*, **72(4)**, 495-500 (2001)). Other lipases of use in this invention include lipoprotein lipase, triacylglycerol lipase, diglyceride lipase, and postheparin lipase.

Esterases

[0372] Esterases can also be used in some embodiments of the invention. Acetylation of cellobiose and cellulose was shown to be catalyzed in aqueous medium in the presence of isopropenyl acetate by an intracellular carboxylesterase from *Arthrobacter viscosus*. (Cui *et al.*, *Enzyme Microb. Technol.*, **24**, 200-208 (1999)). Another group acetylated the amino groups of chitobiose and chitotetraose in an aqueous solution of 3M sodium acetate using a chitin deacetylase from *Colletotrichum lindemuthianum* (Tokuyasu *et al.*, *Carbohydr. Res.*, **322**, 26-31 (1999)). A third group utilized acetylxylan esterase (AcXE) from *Schizophyllum commune* to catalyze acetyl group transfer to methyl β-D-xylopyranoside, methyl β-D-cellobioside, methyl β-D-glucopyranoside, cellotetraose, 2-deoxy-D-glucose, D-mannose, β-

1,4-mannobiose, β-1,4-mannopentaose, β-1,4-mannohexaose, β-1,4-xylobiose, and β-1,4-xylopentaose. (Biely *et al.*, *Biochimica et Biophysica Acta*, **1623**, 62-71 (2003)). Acetylation of secondary alcohols was also achieved by transesterification from vinyl acetate by a feruloyl esterase from *Humicola insolens*. (Hatzakis *et al.*, J. Mol. Catal., B Enzym. **21**, 309-311 (2003). Other esterases of use in this invention include choline esterase, sterol esterase, hydroxycinnamoyl esterase, acetylsalicyclic acid esterase, and polyneuridine esterase.

Acylases

[0373] Acylases can also be used in some embodiments of the invention. Exemplary acylases of use in this invention include aminoacylase I, L-amino-acid acylase, penicillin acylase, acetyl-CoA acylase, acyl-lysine deacylase, aculeacin A acylase, succinyl-CoA acylase, and acetyl-aspartic deaminase.

Acetyltransferases

[0374] In another embodiment of the invention, acyl transfer is accomplished by an acetyltransferase. The use of acetyltransferases in the acylation of saccharides has been previously reported. O-acetylation at the 9 position of sialic acid has been shown to occur from the product of several genes in the COS cell system (Shi *et al.*, *Glycobiology*, **8(2)**, 199-205 (1998)). Maltose O-acetyltransferase (MAT) from *Escherichia coli* is known to catalyze acetyl group transfer to the C6 positions of glucose and maltose. (Leggio *et al.*, *Biochemistry*, **42**, 5225-5235 (2003)). This same group also utilized galactoside acetyltransferase (GAT) to catalyze acetyl group transfer to galactosyl units. Other acetyltransferases of use in this invention include spermidine acetyltransferase, diamine N-acetyltransferase, and sialate O-acetyltransferase.

Sugar Transfer

[0375] In addition to the enzymes discussed above in the context of forming the acyl-linked conjugate, the glycosylation pattern of the conjugate and the starting substrates (*e.g.*, peptides, lipids) can be elaborated, trimmed back or otherwise modified by methods utilizing other enzymes. The methods of remodeling peptides and lipids using enzymes that transfer a sugar donor to an acceptor are discussed in great detail in DeFrees, WO 03/031464 A2, published April 17, 2003. A brief summary of selected enzymes of use in the present method is set forth below.

Glycosyltransferases

[0376] Glycosyltransferases catalyze the addition of activated sugars (donor NDP-sugars), in a step-wise fashion, to a protein, glycopeptide, lipid or glycolipid or to the non-reducing

end of a growing oligosaccharide. N-linked glycopeptides are synthesized via a transferase and a lipid-linked oligosaccharide donor Dol-PP-$NAG_2Glc_3Man_9$ in an en block transfer followed by trimming of the core. In this case the nature of the "core" saccharide is somewhat different from subsequent attachments. A very large number of glycosyltransferases are known in the art.

[0377] For enzymatic saccharide syntheses that involve glycosyltransferase reactions, glycosyltransferase can be cloned, or isolated from any source. Many cloned glycosyltransferases are known, as are their polynucleotide sequences. *See, e.g.*, "The WWW Guide To Cloned Glycosyltransferases," (http://www.vei.co.uk/TGN/gt_guide.htm). Glycosyltransferase amino acid sequences and nucleotide sequences encoding glycosyltransferases from which the amino acid sequences can be deduced are also found in various publicly available databases, including GenBank, Swiss-Prot, EMBL, and others.

[0378] Glycosyltransferases that can be employed in the methods of the invention include, but are not limited to, galactosyltransferases, fucosyltransferases, glucosyltransferases, N-acetylgalactosaminyltransferases, N-acetylglucosaminyltransferases, glucuronyltransferases, sialyltransferases, mannosyltransferases, glucuronic acid transferases, galacturonic acid transferases, and oligosaccharyltransferases. Suitable glycosyltransferases include those obtained from eukaryotes, as well as from prokaryotes.

Fucosyltransferases

[0379] In some embodiments, a glycosyltransferase used in the method of the invention is a fucosyltransferase. Fucosyltransferases are known to those of skill in the art. Exemplary fucosyltransferases include enzymes, which transfer L-fucose from GDP-fucose to a hydroxy position of an acceptor sugar. Fucosyltransferases that transfer non-nucleotide sugars to an acceptor are also of use in the present invention.

[0380] In some embodiments, the acceptor sugar is, for example, the GlcNAc in a Galβ(1→3,4)GlcNAcβ- group in an oligosaccharide glycoside. Suitable fucosyltransferases for this reaction include the Galβ(1→3,4)GlcNAcβ1-α(1→3,4)fucosyltransferase (FTIII E.C. No. 2.4.1.65), which was first characterized from human milk (*see*, Palcic, *et al., Carbohydrate Res.* **190**: 1-11 (1989); Prieels, *et al., J. Biol. Chem.* **256**: 10456-10463 (1981); and Nunez, *et al., Can. J. Chem.* **59**: 2086-2095 (1981)) and the Galβ(1→4)GlcNAcβ-αfucosyltransferases (FTIV, FTV, FTVI) which are found in human serum. FTVII (E.C. No. 2.4.1.65), a sialyl α(2→3)Galβ((1→3)GlcNAcβ fucosyltransferase, has also been

characterized. A recombinant form of the Galβ(1→3,4) GlcNAcβ-α(1→3,4)fucosyltransferase has also been characterized (*see*, Dumas, *et al., Bioorg. Med. Letters* **1**: 425-428 (1991) and Kukowska-Latallo, *et al., Genes and Development* **4**: 1288-1303 (1990)). Other exemplary fucosyltransferases include, for example, α1,2 fucosyltransferase (E.C. No. 2.4.1.69). Enzymatic fucosylation can be carried out by the methods described in Mollicone, *et al., Eur. J. Biochem.* **191**: 169-176 (1990) or U.S. Patent No. 5,374,655. Cells that are used to produce a fucosyltransferase will also include an enzymatic system for synthesizing GDP-fucose.

Galactosyltransferases

[0381] In another group of embodiments, the glycosyltransferase is a galactosyltransferase. Exemplary galactosyltransferases include α(1,3) galactosyltransferases (E.C. No. 2.4.1.151, *see, e.g.*, Dabkowski *et al., Transplant Proc.* **25**:2921 (1993) and Yamamoto *et al. Nature* **345**: 229-233 (1990), bovine (GenBank j04989, Joziasse *et al., J. Biol. Chem.* **264**: 14290-14297 (1989)), murine (GenBank m26925; Larsen *et al., Proc. Nat'l. Acad. Sci. USA* **86**: 8227-8231 (1989)), porcine (GenBank L36152; Strahan *et al., Immunogenetics* **41**: 101-105 (1995)). Another suitable α1,3 galactosyltransferase is that which is involved in synthesis of the blood group B antigen (EC 2.4.1.37, Yamamoto *et al., J. Biol. Chem.* **265**: 1146-1151 (1990) (human)). Yet a further exemplary galactosyltransferase is core Gal-T1.

[0382] Also suitable for use in the methods of the invention are β(1,4) galactosyltransferases, which include, for example, EC 2.4.1.90 (LacNAc synthetase) and EC 2.4.1.22 (lactose synthetase) (bovine (D'Agostaro *et al., Eur. J. Biochem.* **183**: 211-217 (1989)), human (Masri *et al., Biochem. Biophys. Res. Commun.* **157**: 657-663 (1988)), murine (Nakazawa *et al.*, J. Biochem. **104**: 165-168 (1988)), as well as E.C. 2.4.1.38 and the ceramide galactosyltransferase (EC 2.4.1.45, Stahl *et al., J. Neurosci. Res.* **38**: 234-242 (1994)). Other suitable galactosyltransferases include, for example, α1,2 galactosyltransferases (from *e.g.*, *Schizosaccharomyces pombe*, Chapell *et al., Mol. Biol. Cell* **5**: 519-528 (1994)).

Sialyltransferases

[0383] Sialyltransferases are another type of glycosyltransferase that is useful in the recombinant cells and reaction mixtures of the invention. Cells that produce recombinant sialyltransferases will also produce CMP-sialic acid, which is a sialic acid donor for sialyltransferases. Examples of sialyltransferases that are suitable for use in the present invention include ST3Gal III (*e.g.*, a rat or human ST3Gal III), ST3Gal IV, ST3Gal I, ST6Gal

I, ST3Gal V, ST6Gal II, ST6GalNAc I, ST6GalNAc II, and ST6GalNAc III (the sialyltransferase nomenclature used herein is as described in Tsuji *et al., Glycobiology* 6: v-xiv (1996)). An exemplary α(2,3)sialyltransferase referred to as α(2,3)sialyltransferase (EC 2.4.99.6) transfers sialic acid to the non-reducing terminal Gal of a Galβ1→3Glc disaccharide or glycoside. *See,* Van den Eijnden *et al., J. Biol. Chem.* **256:** 3159 (1981), Weinstein *et al., J. Biol. Chem.* **257:** 13845 (1982) and Wen *et al., J. Biol. Chem.* **267:** 21011 (1992). Another exemplary α2,3-sialyltransferase (EC 2.4.99.4) transfers sialic acid to the non-reducing terminal Gal of the disaccharide or glycoside. *see,* Rearick *et al., J. Biol. Chem.* **254:** 4444 (1979) and Gillespie *et al., J. Biol. Chem.* **267:** 21004 (1992). Further exemplary enzymes include Gal-β-1,4-GlcNAc α-2,6 sialyltransferase (*See*, Kurosawa *et al. Eur. J. Biochem.* **219:** 375-381 (1994)).

[0384] Preferably, for glycosylation of carbohydrates of glycopeptides the sialyltransferase will be able to transfer sialic acid to the sequence Galβ1,4GlcNAc-, the most common penultimate sequence underlying the terminal sialic acid on fully sialylated carbohydrate structures (*see,* Table 3).

Table 3: Sialyltransferases which use the Galβ1,4GlcNAc sequence as an acceptor substrate

Sialyltransferase	**Source**	**Sequence(s) formed**	**Ref.**
ST6Gal I	Mammalian	NeuAcα2,6Galβ1,4GlcNAc-	1
ST3Gal III	Mammalian	NeuAcα2,3Galβ1,4GlcNAc- NeuAcα2,3Galβ1,3GlcNAc-	1
ST3Gal IV	Mammalian	NeuAcα2,3Galβ1,4GlcNAc- NeuAcα2,3Galβ1,3GlcNAc-	1
ST6Gal II	Mammalian	NeuAcα2,6Galβ1,4GlcNAc-	
ST6Gal II	photobacterium	NeuAcα2,6Galβ1,4GlcNAc-	2
ST3Gal V	*N. meningitides* *N. gonorrhoeae*	NeuAcα2,3Galβ1,4GlcNAc-	3

1) Goochee *et al., Bio/Technology* **9:** 1347-1355 (1991)
2) Yamamoto *et al., J. Biochem.* **120:** 104-110 (1996)
3) Gilbert *et al., J. Biol. Chem.* **271:** 28271-28276 (1996)

[0385] An example of a sialyltransferase that is useful in the claimed methods is ST3Gal III, which is also referred to as α(2,3)sialyltransferase (EC 2.4.99.6). This enzyme catalyzes

the transfer of sialic acid to the Gal of a Galβ1,3GlcNAc or Galβ1,4GlcNAc glycoside (*see, e.g.*, Wen *et al., J. Biol. Chem.* **267**: 21011 (1992); Van den Eijnden *et al., J. Biol. Chem.* **256:** 3159 (1991)) and is responsible for sialylation of asparagine-linked oligosaccharides in glycopeptides. The sialic acid is linked to a Gal with the formation of an α-linkage between the two saccharides. Bonding (linkage) between the saccharides is between the 2-position of NeuAc and the 3-position of Gal. This particular enzyme can be isolated from rat liver (Weinstein *et al., J. Biol. Chem.* **257**: 13845 (1982)); the human cDNA (Sasaki *et al.* (1993) *J. Biol. Chem.* **268**: 22782-22787; Kitagawa & Paulson (1994) *J. Biol. Chem.* **269**: 1394-1401) and genomic (Kitagawa *et al.* (1996) *J. Biol. Chem.* **271**: 931-938) DNA sequences are known, facilitating production of this enzyme by recombinant expression. In a preferred embodiment, the claimed sialylation methods use a rat ST3Gal III.

[0386] Other exemplary sialyltransferases of use in the present invention include those isolated from *Campylobacter jejuni*, including the α(2,3). *See*, e.g, WO99/49051.

[0387] Sialyltransferases other those listed in Table 3, are also useful in an economic and efficient large-scale process for sialylation of commercially important glycopeptides. As a simple test to find out the utility of these other enzymes, various amounts of each enzyme (1-100 mU/mg protein) are reacted with asialo-α_1 AGP (at 1-10 mg/ml) to compare the ability of the sialyltransferase of interest to sialylate glycopeptides relative to either bovine ST6Gal I, ST3Gal III or both sialyltransferases. Alternatively, other glycopeptides or glycopeptides, or N-linked oligosaccharides enzymatically released from the peptide backbone can be used in place of asialo-α_1 AGP for this evaluation. Sialyltransferases with the ability to sialylate N-linked oligosaccharides of glycopeptides more efficiently than ST6Gal I are useful in a practical large-scale process for peptide sialylation.

[0388] **FIG. 2** provides a listing of exemplary sialyltransferases of use in the present invention.

GalNAc transferases

[0389] N-acetylgalactosaminyltransferases are of use in practicing the present invention, particularly for binding a GalNAc moiety to an amino acid of the O-linked glycosylation site of the peptide. Suitable N-acetylgalactosaminyltransferases include, but are not limited to, α(1,3) N-acetylgalactosaminyltransferase, β(1,4) N-acetylgalactosaminyltransferases (Nagata *et al., J. Biol. Chem.* **267**: 12082-12089 (1992) and Smith *et al., J. Biol Chem.* **269**: 15162

(1994)) and polypeptide N-acetylgalactosaminyltransferase (Homa *et al., J. Biol. Chem.* **268**: 12609 (1993)).

[0390] Production of proteins such as the enzyme GalNAc T_{I-XX} from cloned genes by genetic engineering is well known. See, *e.g.*, U.S. Pat. No. 4,761,371. One method involves collection of sufficient samples, then the amino acid sequence of the enzyme is determined by N-terminal sequencing. This information is then used to isolate a cDNA clone encoding a full-length (membrane bound) transferase which upon expression in the insect cell line Sf9 resulted in the synthesis of a fully active enzyme. The acceptor specificity of the enzyme is then determined using a semiquantitative analysis of the amino acids surrounding known glycosylation sites in 16 different proteins followed by in vitro glycosylation studies of synthetic peptides. This work has demonstrated that certain amino acid residues are overrepresented in glycosylated peptide segments and that residues in specific positions surrounding glycosylated serine and threonine residues may have a more marked influence on acceptor efficiency than other amino acid moieties.

Cell-Bound Glycosyltransferases

[0391] In another embodiment, the enzymes utilized in the method of the invention are cell-bound glycosyltransferases. Although many soluble glycosyltransferases are known (see, for example, U.S. Pat. No. 5,032,519), glycosyltransferases are generally in membrane-bound form when associated with cells. Many of the membrane-bound enzymes studied thus far are considered to be intrinsic proteins; that is, they are not released from the membranes by sonication and require detergents for solubilization. Surface glycosyltransferases have been identified on the surfaces of vertebrate and invertebrate cells, and it has also been recognized that these surface transferases maintain catalytic activity under physiological conditions. However, the more recognized function of cell surface glycosyltransferases is for intercellular recognition (Roth, MOLECULAR APPROACHES to SUPRACELLULAR PHENOMENA, 1990).

[0392] Methods have been developed to alter the glycosyltransferases expressed by cells. For example, Larsen *et al., Proc. Natl. Acad. Sci. USA* **86**: 8227-8231 (1989), report a genetic approach to isolate cloned cDNA sequences that determine expression of cell surface oligosaccharide structures and their cognate glycosyltransferases. A cDNA library generated from mRNA isolated from a murine cell line known to express UDP-galactose:.β.-D-galactosyl-1,4-N-acetyl-D-glucosaminide α-1,3-galactosyltransferase was transfected into

COS-1 cells. The transfected cells were then cultured and assayed for α 1-3 galactosyltransferase activity.

[0393] Francisco *et al., Proc. Natl. Acad. Sci. USA* **89**: 2713-2717 (1992), disclose a method of anchoring β-lactamase to the external surface of *Escherichia coli*. A tripartite fusion consisting of (i) a signal sequence of an outer membrane protein, (ii) a membrane-spanning section of an outer membrane protein, and (iii) a complete mature β-lactamase sequence is produced resulting in an active surface bound β-lactamase molecule. However, the Francisco method is limited only to procaryotic cell systems and as recognized by the authors, requires the complete tripartite fusion for proper functioning.

Sulfotransferases

[0394] The invention also provides methods for producing peptides that include sulfated molecules, including, for example sulfated polysaccharides such as heparin, heparan sulfate, carragenen, and related compounds. Suitable sulfotransferases include, for example, chondroitin-6-sulphotransferase (chicken cDNA described by Fukuta *et al., J. Biol. Chem.* **270**: 18575-18580 (1995); GenBank Accession No. D49915), glycosaminoglycan N-acetylglucosamine N-deacetylase/N-sulphotransferase 1 (Dixon *et al., Genomics* **26**: 239-241 (1995); UL18918), and glycosaminoglycan N-acetylglucosamine N-deacetylase/N-sulphotransferase 2 (murine cDNA described in Orellana *et al., J. Biol. Chem.* **269**: 2270-2276 (1994) and Eriksson *et al., J. Biol. Chem.* **269**: 10438-10443 (1994); human cDNA described in GenBank Accession No. U2304).

Glycosidases

[0395] This invention also encompasses the use of wild-type and mutant glycosidases. Mutant β-galactosidase enzymes have been demonstrated to catalyze the formation of disaccharides through the coupling of an α-glycosyl fluoride to a galactosyl acceptor molecule. (Withers, U.S. Pat. No. 6,284,494; issued Sept. 4, 2001). Other glycosidases of use in this invention include, for example, β-glucosidases, β-galactosidases, β-mannosidases, β-acetyl glucosaminidases, β-N-acetyl galactosaminidases, β-xylosidases, β-fucosidases, cellulases, xylanases, galactanases, mannanases, hemicellulases, amylases, glucoamylases, α-glucosidases, α-galactosidases, α-mannosidases, α-N-acetyl glucosaminidases, α-N-acetyl galactose-aminidases, α-xylosidases, α-fucosidases, and neuraminidases/sialidases.

Immobilized Enzymes

[0396] The present invention also provides for the use of enzymes that are immobilized on a solid and/or soluble support. In an exemplary embodiment, there is provided a

glycosyltransferase that is conjugated to a PEG via an intact glycosyl linker according to the methods of the invention. The PEG-linker-enzyme conjugate is optionally attached to solid support. The use of solid supported enzymes in the methods of the invention simplifies the work up of the reaction mixture and purification of the reaction product, and also enables the facile recovery of the enzyme. The glycosyltransferase conjugate is utilized in the methods of the invention. Other combinations of enzymes and supports will be apparent to those of skill in the art.

Glycosylation by Recombinant Methods

[0397] FGF peptide conjugates may also be prepared intracellularly by recombinant means. A polynucleotide sequence encoding a FGF, which comprises at least one newly introduced N- or O-linked glycosylation site, may be transfected into a suitable host cell line, *e.g.*, a eukaryotic cell line derived from yeast, insect, or mammalian origin. The Fibroblast Growth Factor recombinantly produced from such a cell line is glycosylated by the host cell glycosylation machinery.

Purification of FGF peptide conjugates

[0398] The FGF peptide conjugate produced by the above processes is preferably purified before use. Standard, well known techniques such as thin or thick layer chromatography, column chromatography, ion exchange chromatography, or membrane filtration can be used. It is preferred to use membrane filtration, more preferably utilizing a reverse osmotic membrane, or one or more column chromatographic techniques for the recovery as is discussed hereinafter and in the literature cited herein.

[0399] If the FGF peptide conjugate is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration; optionally, the protein may be concentrated with a commercially available protein concentration filter, followed by separating the polypeptide variant from other impurities by one or more steps selected from immunoaffinity chromatography, ion-exchange column fractionation (*e.g.*, on diethylaminoethyl (DEAE) or matrices containing carboxymethyl or sulfopropyl groups), chromatography on Blue-Sepharose, CM Blue-Sepharose, MONO-Q, MONO-S, lentil lectin-Sepharose, WGA-Sepharose, Con A-Sepharose, Ether Toyopearl, Butyl Toyopearl, Phenyl Toyopearl, SP-Sepharose, or protein A Sepharose, SDS-PAGE chromatography, silica chromatography, chromatofocusing, reverse phase HPLC (*e.g.*, silica gel with appended aliphatic groups), gel

filtration using, *e.g.*, Sephadex molecular sieve or size-exclusion chromatography, chromatography on columns that selectively bind the polypeptide, and ethanol or ammonium sulfate precipitation.

[0400] A FGF peptide conjugate produced in culture is usually isolated by initial extraction from cells, cell lysate, culture media, *etc.*, followed by one or more concentration, salting-out, aqueous ion-exchange, or size-exclusion chromatography steps. Additionally, the glycoprotein may be purified by affinity chromatography. Finally, HPLC may be employed for final purification steps.

[0401] A protease inhibitor, *e.g.*, methylsulfonylfluoride (PMSF) may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

[0402] In some cases, supernatants from systems that produce the FGF peptide conjugates of the invention are first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate may be applied to a suitable purification matrix. For example, a suitable affinity matrix may comprise a ligand for the peptide, a lectin or antibody molecule bound to a suitable support. Alternatively, an anion-exchange resin may be employed, for example, a matrix or substrate having pendant DEAE groups. Suitable matrices include acrylamide, agarose, dextran, cellulose, or other types commonly employed in protein purification. Also, a cation-exchange step may be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are particularly preferred.

[0403] Finally, one or more RP-HPLC steps employing hydrophobic RP-HPLC media, *e.g.*, silica gel having pendant methyl or other aliphatic groups, may be employed to further purify a FGF peptide conjugate. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a glycoprotein.

[0404] The FGF peptide conjugate of the invention resulting from a large-scale fermentation may be purified by methods analogous to those disclosed by Urdal *et al., J. Chromatog.* **296**: 171 (1984). This reference describes two sequential, RP-HPLC steps for purification of recombinant human IL-2 on a preparative HPLC column. Alternatively, techniques such as affinity chromatography, may be utilized to purify the glycoprotein.

[0405] Following the production and, preferably, purification of a glycosylated mutant Fibroblast Growth Factor, the biological functions of the glycoprotein are tested using several methods known in the art. The functional assays are based on various characteristics of Fibroblast Growth Factor.

Pharmaceutical Composition and Administration

[0406] The FGF peptide conjugates having desired oligosaccharide determinants described above can be used as therapeutics for treating a variety of diseases and conditions related to deficiency in growth hormone. Growth-related conditions that can be treated with the FGF peptide conjugates of the present invention include: dwarfism, short-stature in children and adults, cachexia/muscle wasting, general muscular atrophy, and sex chromosome abnormality (*e.g.*, Turner's Syndrome). Other conditions may be treated using the FGF peptide conjugates of the present invention include: short-bowel syndrome, lipodystrophy, osteoporosis, uraemaia, burns, female infertility, bone regeneration, general diabetes, type II diabetes, osteo-arthritis, chronic obstructive pulmonary disease (COPD), and insomia. The FGF peptide conjugates of the invention may also be used to promote various healing processes, *e.g.*, general tissue regeneration, bone regeneration, and wound healing, or as a vaccine adjunct. Thus, the present invention also provides pharmaceutical compositions comprising an effective amount of FGF peptide conjugate, which is produced according to the methods described above.

[0407] Pharmaceutical compositions of the invention are suitable for use in a variety of drug delivery systems. Suitable formulations for use in the present invention are found in *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). For a brief review of methods for drug delivery, *see*, Langer, *Science* **249**: 1527-1533 (1990).

[0408] The pharmaceutical compositions are intended for parenteral, intranasal, topical, oral, or local administration, such as by subcutaneous injection, aerosol inhalation, or transdermal adsorption, for prophylactic and/or therapeutic treatment. Commonly, the pharmaceutical compositions are administered parenterally, *e.g.*, subcutaneously or intravenously. Thus, the invention provides compositions for parenteral administration which comprise the FGF peptide conjugate dissolved or suspended in an acceptable carrier, preferably an aqueous carrier, *e.g.*, water, buffered water, saline, PBS and the like. The compositions may also contain detergents such as Tween 20 and Tween 80; stablizers such as

mannitol, sorbitol, sucrose, and trehalose; and preservatives such as EDTA and m-cresol. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents, detergents and the like.

[0409] These compositions may be sterilized by conventional sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous carrier prior to administration. The pH of the preparations typically will be between 3 and 11, more preferably from 5 to 9, and most preferably from 7 and 8.

[0410] The compositions containing the FGF peptide conjugates can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a disease or condition related to growth hormone deficiency, in an amount sufficient to cure or at least partially arrest the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend on the severity of the disease or condition and the weight and general state of the patient, but generally range from about 0.1 mg to about 2,000 mg of FGF peptide conjugates per day for a 70 kg patient, with dosages of from about 5 mg to about 200 mg of the compounds per day being more commonly used.

[0411] In prophylactic applications, compositions containing the FGF peptide conjugate of the invention are administered to a patient susceptible to or otherwise at risk of a particular disease. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight, but generally range from about 0.1 mg to about 1,000 mg per 70 kilogram patient, more commonly from about 5 mg to about 200 mg per 70 kg of body weight.

[0412] Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of the FGF peptide conjugate of this invention sufficient to effectively treat the patient.

EXAMPLES

[0413] The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of non-critical parameters that could be changed or modified to yield essentially similar results. Though the method is exemplified by reference to FGF-20 and FGF-21, those of skill will appreciate that glycosylation sites can be incorporated into the peptide sequences of other FGFs, *e.g.* FGF-9 and FGF-18, in the manner set forth below.

Fibroblast Growth Factor-20 sequence information

[0414] A Fibroblast Growth Factor-20 sequence displaying the different regions of the protein is shown in Table 5. The wild-type FGF-20 is thought to be un-glycosylated and may be produced in *Escherichia coli* as a therapeutic. The amino acid sequence is shown in Table 4, below.

Table 4. Human Fibroblast Growth Factor-20, (SEQ ID NO:1)

MAPLAEVGGF LGGLEGLGQQ VGSHFLLPPA GERPPLLGER RSAAERSARG
GPGAAQLAHL HGILRRRQLY CRTGFHLQIL PDGSVQGTRQ DHSLFGILEF
ISVAVGLVSI RGVDSGLYLG MNDKGELYGS EKLTSECIFR EQFEENWYNT
YSSNIYKHGD TGRRYFVALN KDGTPRDGAR SKRHQKFTHF LPRPVDPERV
PELYKDLLMY T

[0415] Regions of FGF-20 that are suited to mutation for the purpose of creating glycosylation sites are shown in Table 5. These regions are indicated either in bold, or in italics when one region is contiguous to another.

Table 5: Wild-type Human FGF-20 Sequence Showing Different Protein Regions

MAPLAEVGGF LGGLEGLGQQ VGSHFLLPPA GERPPLLGER RS*AAERSARG GPGAAQLAHL*

region 1 region 2 region 3

1.....10.....20.....30.....40.....50.....60

HGILRRRQLY CR**TGFHLQIL** PDGSVQGTRQ DHSLFGILEF ISVAVGLVSI RGVDSGLYLGMN

region 4

61.....70.....80.....90.....100.....110.....120...

DKGELYGSEKLTSECIFR EQFEENWYNTYSSNIYKHGD TGRRYFVA**LN KDGTPRDGAR SKRH**

region 5

123... 130.....140.....150.....160.....170.....180...

QKFTHF LPRPVDPERV PELYKDL*LMY T*

region 6 region 7

185...190.....200.....210.211

Fibroblast Growth Factor-21 sequence information

[0416] A Fibroblast Growth Factor-21 sequence displaying the different regions of the protein is shown in Table 7. The wild-type FGF-21 is thought to be un-glycosylated and may be produced in *Escherichia coli* as a therapeutic. The amino acid sequence is shown in Table 6, below.

Table 6. Human Fibroblast Growth Factor-21, (SEQ ID NO:146)

MHP IPDSSPLLQF GGQVRQRYLY TDDAQQTEAH LEIREDGTVG GAADQSPESL LQLKALKPGV IQILGVKTSR FLCQRPDGAL YGSLHFDPEA CSFRELLLED GYNVYQSEAH GLPLHLPGNK SPHRDPAPRG PARFLPLPGL PPALPEPPGI LAPQPPDVGS SDPLSMVGPS QGRSPSYAS

[0417] Regions of FGF-21 that are suited to mutation for the purpose of creating glycosylation sites are shown in Table 7. These regions are indicated either in bold, or in italics when one region is contiguous to another.

Table 7: Wild-type Human FGF-21 Sequence Showing Different Protein Regions

MHPIPDSS*PLLQF*GGQVRQRYLYTDDAQQTEAHLEIREDGTVGGAADQSPESLL

region 1 region 2 region 3

1.....10.....20.....30.....40.....50.....

QLKALK**PGVIQ**ILGVKTSRFLCQ**RPDGA**LYG**SLHFDP**EACSFRELLLEDGYNVYQS

region 4 region 5 region 6

55.....60.....70.....80.....90.....100.....110

EAHGLPLHLPGNKSPHRDPAPRGPARFLPLPGLPPALP**EPPGILAPQ**PP

region 7 region 8

111.....120.....130.....140.....150.....159

DVGSSDP*LSMVGP*SQGRSPSYAS

region 9 region 10 region 11

160.....170.....180.182

[0418] FGF or mutated FGF can be glycosylated or glycoconjugated (see WO 03/31464, incorporated herein by reference). Preferably, a mutated FGF is glycoPEGylated, wherein a polyethylene glycol (PEG) moiety is conjugated to the mutated FGF polypeptide via a glycosyl linkage (see WO 03/31464, incorporated herein by reference). GlycoPEGylation of the FGF is expected to result in improved biophysical properties that may include but are not limited to improved half-life, improved area under the curve (AUC) values, reduced clearance, and reduced immunogenicity.

EXAMPLE 1

[0419] Exemplary regions on FGF-20 that are suited for the introduction of glycosylation sites by mutation are shown in Table 5, above. In all cases, the N-terminal Met may be present or absent on any FGF mutant. The numbering of the amino acid residues is based on the initial unmodified sequence in which the left most residue, methionine, is numbered as position 1. To highlight how the mutant sequence differs in respect to the unmodified sequence, the numbering of unmodified amino acids as they appear in the sequences below remains unchanged following the modification. More than one of the described sequence modifications may be present in an FGF mutant of the present invention. Specifically, the preferred regions for introduction of mutations to create a glycosylation site(s) not present in the wild-type peptide are the nucleotide sequences that encode: amino acids 1-7 (REGION 1; SEQ ID NO:2), amino acids 20-42 (REGION 2; SEQ ID NO:3), amino acids 43-60 (REGION 3; SEQ ID NO:4), amino acids 73-90 (REGION 4; SEQ ID NO:5), amino acids 159-174 (REGION 5; SEQ ID NO:6), amino acids 177-198 (REGION 6; SEQ ID NO:7) or amino acids 199-201 (REGION 7; SEQ ID NO:8) of the wild-type FGF amino acid sequence (see Table 5) can be mutated so that either an N-linked or an O-linked glycosylation site is introduced into the resulting mutated FGF-20 polypeptide.

[0420] The following example describes amino acid sequence mutations introducing N-linked *e.g.*, asparagine residues, and O-linked glycosylation sites, *e.g.*, serine or threonine residues, into a preferably proline-containing site of a wild-type Fibroblast Growth Factor-20 sequence or any modified version thereof.

1. Region 1

[0421] In the Region 1 mutants, the N-terminus of a wild-type FGF-20, MAP^3LAEV; SEQ ID NO:2, is replaced with $MXY_aZ_bP^3BJO1234$, wherein 1, 2, 3, 4, X, Y, Z, B, J and O are

independently selected from any uncharged amino acid, or glutamic acid (E), and wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. The symbols a and b independently represent 0 or 1. To clarify, sequences designated as SEQ ID NO:12-14, 338-344 contain amino acid insertions between P^3 and L^4 of the native FGF-20 sequence. Preferred examples include:

MAPTP^{3}LAEV;	SEQ ID NO:9
MVTP^{3}LAEV;	SEQ ID NO:10
MAP^{3}TTEV;	SEQ ID NO:11
MAP^{3}TQGAMPL^{4}AEV;	SEQ ID NO:12
MAP^{3}TSSL^{4}AEV;	SEQ ID NO:13
MAP^{3}TALPL^{4}AEV;	SEQ ID NO:14
MAP^{3}TQAPL^{4}AEV;	SEQ ID NO:338
MAP^{3}TEIPL^{4}AEV;	SEQ ID NO:339
MAP^{3}TINTPL^{4}AEV;	SEQ ID NO:340
MAP^{3}TINTL^{4}AEV;	SEQ ID NO:341
MAP^{3}TTVSL^{4}AEV;	SEQ ID NO:342
MAP^{3}TQEVL^{4}AEV;	SEQ ID NO:343
MAP^{3}TQAVL^{4}AEV;	SEQ ID NO:344

2. Region 2

[0422] In these mutants, the wild-type QVGSHFLLP^{28}P^{29}A^{30}GERPPLLGERRS; SEQ ID NO:3, is subdivided into three regions: Region 2(a) VGSHFLLP^{28}P^{29}A^{30}GERPP, SEQ ID NO:15; Region 2(b) P^{28}P^{29}AGERPP, SEQ ID NO:16; and Region 2(c) P^{34}P^{35}PLLGERRS, SEQ ID NO:17. Mutations in each region are considered separately below.

Region 2(a): in these mutants the wild-type VGSHFLLP^{28}P^{29}A^{30}GERPP (SEQ ID NO:15) is replaced with 1234XYZ P^{28}P^{29}A^{30} wherein 1, 2, 3, 4, X, Y, Z, are independently selected from any uncharged amino acid, or glutamic acid (E), wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site . The symbols a and b independently represent 0 or 1. Preferred mutations include:

TET P^{28}P^{29}A^{30}GERPP;	SEQ ID NO:18
GTET P^{28}P^{29}A^{30}GERPP;	SEQ ID NO:19

VGTET $P^{28}P^{29}A^{30}$GERPP; SEQ ID NO:20
TGT $P^{28}P^{29}$AEERPP; SEQ ID NO:21
TAT $P^{28}P^{29}$ AEERPP; SEQ ID NO:22

Region 2(b): in these mutants the wild-type $P^{28}P^{29}A^{30}$GERPP (SEQ ID NO:16) is replaced with $P^{28}P^{29}1234(5)_a$PP wherein 1, 2, 3, 4, X, Y, Z, are as described for Region 2(a). Preferred mutations include:

P^{28} P^{29}TGEAPP; SEQ ID NO:23
P^{28} P^{29}TGEVPP; SEQ ID NO:24
P^{28} P^{29}TQGAPP; SEQ ID NO:25
P^{28} P^{29}ATVAPP; SEQ ID NO:26
P^{28} P^{29}ATILPP; SEQ ID NO:27
P^{28} P^{29}AGTAPP; SEQ ID NO:28
P^{28} P^{29}TQGAMPP; SEQ ID NO:29
P^{28} P^{29}GSTAPP; SEQ ID NO:30
P^{28} P^{29}AGTSPP; SEQ ID NO:31
P^{28} P^{29}AGETPP; SEQ ID NO:32
P^{28} P^{29}ATETPP; SEQ ID NO:33
P^{28} P^{29}GTETPP; SEQ ID NO:34
P^{28} P^{29}TGERPP; SEQ ID NO:35
$P^{28}P^{29}$TINTPP; SEQ ID NO:345
$P^{28}P^{29}$ TTVSPP; SEQ ID NO:346
$P^{28}P^{29}$ TQALPP; SEQ ID NO:347

Region 2(c): in these mutants the wild-type $P^{34}P^{35}$PLLGERRS (SEQ ID NO:17) is replaced with $P^{34}P^{35}$123456 wherein 1, 2, 3, 4, 5, 6, are independently selected from any uncharged amino acid, or glutamic acid (E), and wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. Preferred mutations include:

$P^{34}P^{35}$TQGAMP; SEQ ID NO:36
$P^{34}P^{35}$TQGAMRS; SEQ ID NO:37
$P^{34}P^{35}$TQGAMAS; SEQ ID NO:38
$P^{34}P^{35}$TQGAMFS; SEQ ID NO:39

$P^{34}P^{35}$TSSSTRS;	SEQ ID NO:40
$P^{34}P^{35}$TSSSTKS;	SEQ ID NO:41
$P^{34}P^{35}$TGERRS;	SEQ ID NO:42
$P^{34}P^{35}$TTGVRRS;	SEQ ID NO:43
$P^{34}P^{35}$TTGEARS;	SEQ ID NO:44
$P^{34}P^{35}$TAGERRS;	SEQ ID NO:45
$P^{34}P^{35}$TINTRRS;	SEQ ID NO:348
$P^{34}P^{35}$TTVSRRS;	SEQ ID NO:349

3. Region 3

[0423] In these mutants, the amino acid sequence surrounding P^{52}, AAERSARGGP^{52}GAAQLAHL; SEQ ID NO:4, is subdivided into two regions; Region 3(a) RSARGGP^{52}; SEQ ID NO:46 and Region 3(b) P^{52}GAAQLA, SEQ ID NO:47. Mutations in each region are considered separately, below.

Region 3(a): in these mutants the wild-type RSARGG P^{52} (SEQ ID NO:46) is replaced with 123456P^{52} wherein 1, 2, 3, 4, 5, 6, are independently selected from any uncharged amino acid, or glutamic acid (E), wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. Preferred examples include:

RSATETP^{52};	SEQ ID NO:48
RSGTETP^{52};	SEQ ID NO:49
RSGTETP^{52};	SEQ ID NO:50
RVGTETP^{52};	SEQ ID NO:51
GVGTETP^{52};	SEQ ID NO:52
GSATETP^{52};	SEQ ID NO:53
GVGVTETP^{52};	SEQ ID NO:54
GVTETP^{52};	SEQ ID NO:55
QTELP^{52};	SEQ ID NO:56
GVTSAP^{52};	SEQ ID NO:57
SVVTP^{52};	SEQ ID NO:58

Region 3(b): in these mutants the wild-type P^{52}GAAQLA (SEQ ID NO:47) is replaced with P^{52}123456 wherein 1, 2, 3, 4, 5, 6, are independently selected from any uncharged amino acid, or glutamic acid (E), wherein at least one is threonine (T) or serine (S), and is a substrate for

GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. Preferred examples include:

P^{52}TGAQLA;	SEQ ID NO:59
P^{52}TQGAMP;	SEQ ID NO:60
P^{52}TQGAMA;	SEQ ID NO:61
P^{52}TTAQLA;	SEQ ID NO:62
P^{52}GATQLA;	SEQ ID NO:63
P^{52}TSSSTA;	SEQ ID NO:64
P^{52}TSSSLA;	SEQ ID NO:65
P^{52}TINTLA;	SEQ ID NO:350
P^{52}TTVSLA;	SEQ ID NO:351
P^{52}TQAQLA;	SEQ ID NO:352

4. Region 4

[0424] In these mutants, the wild-type TGFHLQIL P^{81}DGSVQGTRQ; SEQ ID NO:5, is subdivided into three regions; Region 4(a) HLQILP^{81}; SEQ ID NO:66; Region 4(b) P^{81}DGSVQGT; SEQ ID NO:67; and Region 4(c) P^{81}NGS SEQ ID NO:68. Mutations in each region are considered separately below.

Region 4(a): in these mutants the wild-type HLQILP^{81} (SEQ ID NO:66) is replaced with 12345 P^{81} wherein 1, 2, 3, 4, 5, are independently selected from any uncharged amino acid, or glutamic acid (E), wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. Preferred examples include:

QTELP^{81};	SEQ ID NO:69
LIVTP^{81};	SEQ ID NO:70
LTELP^{81};	SEQ ID NO:71
LTELP^{81};	SEQ ID NO:72
GVTSAP^{81};	SEQ ID NO:73
HLTETP^{81};	SEQ ID NO:74
VLTETP^{81};	SEQ ID NO:75
VGTETP^{81};	SEQ ID NO:76
VGVGTETP^{81};	SEQ ID NO:77
VTSAP^{81};	SEQ ID NO:78

VSTP^{81};	SEQ ID NO:79
EATP^{81};	SEQ ID NO:80

Region 4(b): in these mutants the wild-type P^{81}DGSVQGT (SEQ ID NO:67) is replaced with P^{81}12345GT wherein 1, 2, 3, 4 and 5 are independently selected from any uncharged amino acid, or glutamic acid (E), wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. Preferred examples include:

P^{81}TGSVGT;	SEQ ID NO:81
P^{81}TQGVQGT;	SEQ ID NO:82
P^{81}TGSVGPGT;	SEQ ID NO:83
P^{81}TQGAMPGT;	SEQ ID NO:84
P^{81}TTSVQGT;	SEQ ID NO:85
P^{81}TTAVQGT;	SEQ ID NO:86
P^{81}TINTQGT;	SEQ ID NO:353
P^{81}TTVSQGT;	SEQ ID NO:354

Region 4(c): in these mutants the wild-type P^{81}DGS (SEQ ID NO:68) is mutated to create an N-linked glycosylation site. Preferred examples include:

IL P^{81}NGSVH;	SEQ ID NO:87
IF P^{81}NGSV;	SEQ ID NO:88
P^{81}NGT;	SEQ ID NO:89
L P^{81}NGTVH;	SEQ ID NO:90
P^{81}NGTV;	SEQ ID NO:91
IL P^{81}NGT;	SEQ ID NO:92
QIL P^{81}NGT;	SEQ ID NO:93
QIL P^{81}NGTVH;	SEQ ID NO:94

5. Region 5

[0425] In these mutants the wild-type LN KDGTP^{175}RDGAR SKRH, SEQ ID NO:6 is replaced with 12345 P^{175}67891011 wherein 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, are independently selected from any uncharged amino acid, or glutamic acid (E), wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. Preferred examples include:

LNVTETP^{175}RDGARSKRH;	SEQ ID NO:95
LNVTET P^{175}DDGARSKRH;	SEQ ID NO:96
LNVTET P^{175}LDGARSKRH;	SEQ ID NO:97
LNAITT P^{175}RDGARSKRH;	SEQ ID NO:98
LNAITT P^{175}LDGARSKRH;	SEQ ID NO:99
LNQEAT P^{175}LDGARSKRH;	SEQ ID NO:100
LNQTEL P^{175}LDGARSKRH;	SEQ ID NO:101
LNQTEL P^{175}ADGARSKRH;	SEQ ID NO:102
LNKDGT P^{175}TDGARSKRH;	SEQ ID NO:103
LNKDGT P^{175}TSGARSKRH;	SEQ ID NO:104
LNKDGT P^{175}TDGAASKRH;	SEQ ID NO:105
LNKDGT P^{175}TSGAASKRH;	SEQ ID NO:106
LNKDGT P^{175}TQGAMPKRH;	SEQ ID NO:107
LNKDGT P^{175}TQGAMSKRH;	SEQ ID NO:108
LNKDGT P^{175}TTTARSKRH;	SEQ ID NO:109
LN KDGTP^{175}TINTRSKRH;	SEQ ID NO:355
LN KDGTP^{175}TINTSSKRH;	SEQ ID NO:356
LN KDGTP^{175}TTVSRSKRH;	SEQ ID NO:357
LN KDGTP^{175}TTVSASKRH;	SEQ ID NO:358

6. Region 6

[0426] In these mutants, the wild-type sequence, FTHFL P^{192}RPVD P^{197}ERVP^{201}ELYKDLL; SEQ ID NO:7, is subdivided into two regions; Region 6(a) LP^{192}RPVD P^{197}ERV P^{201}ELYKD; SEQ ID NO:110 and Region 6(b) P^{197}ERVP^{201}ELYKD, SEQ ID NO:111. Mutations in each region are considered separately, below.

Region 6(a): Region 6(a) in these mutants the wild-type LP^{192}RPVD P^{197}ERVP^{201}ELYKD (SEQ ID NO:110) is replaced with P^{192}1P23 P^{197} wherein 1, 2, 3, are independently selected from any uncharged amino acid, or glutamic acid (E), wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. Preferred examples include:

LP^{192}APTD P^{197}ERVP^{201}ELYKD;	SEQ ID NO:112
LP^{192}NPTA P^{197}ERVP^{201}ELYKD;	SEQ ID NO:113
LP^{192}RPTA P^{197}ERVP^{201}ELYKD;	SEQ ID NO:114

$LP^{192}APTQ\ P^{197}ERVP^{201}ELYKD$;	SEQ ID NO:115
$LP^{192}TPVD\ P^{197}ERVP^{201}ELYKD$;	SEQ ID NO:116
$LP^{192}TPSD\ P^{197}ERVP^{201}ELYKD$;	SEQ ID NO:117
$LP^{192}VPTD\ P^{197}ERVP^{201}ELYKD$;	SEQ ID NO:118
$LP^{192}TPAD\ P^{197}ERVP^{201}ELYKD$;	SEQ ID NO:119

Region 6(b): Region 6(b) in these mutants the wild-type $P^{197}ERVP^{201}ELYKD$ (SEQ ID NO:111) is replaced with $P^{197}123P^{201}45678$ wherein 1, 2, 3, 4, 5, 6, 7, 8, are independently selected from any uncharged amino acid, or glutamic acid (E), wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. Preferred examples include:

$P^{197}TAS\ P^{201}ELYKD$;	SEQ ID NO:120
$P^{197}TAS\ P^{201}ALYKD$;	SEQ ID NO:121
$P^{197}NTL\ P^{201}ELYKD$;	SEQ ID NO:122
$P^{197}ETV\ P^{201}ELYKD$;	SEQ ID NO:123
$P^{197}QET\ P^{201}ELYKD$;	SEQ ID NO:124
$P^{197}TQG\ P^{201}ELYKD$;	SEQ ID NO:125
$P^{197}TQG\ P^{201}ALYKD$;	SEQ ID NO:126
$P^{197}QGT\ P^{201}ALYKD$;	SEQ ID NO:127
$P^{197}ATE\ P^{201}ELYKD$;	SEQ ID NO:128
$P^{197}TTQ\ P^{201}ELYKD$;	SEQ ID NO:129
$P^{197}TTE\ P^{201}ELYKD$;	SEQ ID NO:130
$P^{197}ERVP^{201}TLYKD$;	SEQ ID NO:131
$P^{197}ERVP^{201}TLYAD$;	SEQ ID NO:132
$P^{197}ERVP^{201}TQGAD$;	SEQ ID NO:133
$P^{197}ERVP^{201}TQGAMP$;	SEQ ID NO:134
$P^{197}ERVP^{201}TQGA$;	SEQ ID NO:135
$P^{197}TQAP^{201}ELYKD$;	SEQ ID NO:359
$P^{197}TEIP^{201}ELYKD$;	SEQ ID NO:360

7. Region 7

[0427] In these mutants the wild-type $L^{208}MY\ T^{211}$ (SEQ ID NO: 8) is replaced with $123(4)_a(5)_b(6)_c(x)$ wherein 1, 2, 3, 4, 5, are independently selected from any uncharged amino

acid, or glutamic acid (E), wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. The symbols a, b, and c independently represent 0 or 1, and (x) is selected from OH, NH_2, glycine, alanine, leucine, and asparagine. Preferred examples include:

$L^{208}MY\ T^{211}P(x)$;	SEQ ID NO:136
$L^{208}TE\ T^{211}P(x)$;	SEQ ID NO:137
$VTE\ T^{211}P(x)$;	SEQ ID NO:138
$GVTE\ T^{211}PL(x)$;	SEQ ID NO:139
$PELYVGVTC\ T^{211}PL(x)$;	SEQ ID NO:140
$L^{208}MY\ T^{211}$ (x);	SEQ ID NO:141
$L^{208}MY\ T^{211}PTASP$;	SEQ ID NO:142
$L^{208}MY\ T^{211}PATEP$;	SEQ ID NO:143
$L^{208}MY\ T^{211}PTP(x)$;	SEQ ID NO:144
$L^{208}MY\ T^{211}PTAP(x)$;	SEQ ID NO:145

[0428] The numbering of the amino acid residues is based on the initial unmodified sequence in which the most N-terminal residue is numbered 1. The numbering of unmodified amino acids remains unchanged following the modification. More than one of the above described sequence modifications may be present in a FGF mutant of the present invention.

EXAMPLE 2

[0429] A library of FGF-20 peptides each with one potential O-linked glycosylation site as disclosed in Example 1, is expressed in *E. coli* or by using *in vitro* translation methods. Protein is purified using either a heparin binding or IMAC capture method and tested by for *in vitro* biological activity. Those protein sequences that retain *in vitro* activity are tested as acceptors for GlycoPEGylation. GlycoPEGylated FGF-20's (40 kDa branched) are purified for further biological evaluation as outlined above.

EXAMPLE 3

[0430] Exemplary regions on FGF-21 that are suited for the introduction of glycosylation sites by mutation are shown in Table 7, above. In all cases, the N-terminal Met may be present or absent on any FGF mutant. The numbering of the amino acid residues is based on

the initial unmodified sequence in which the left most residue, methionine, is numbered as position 1. To highlight how the mutant sequence differs in respect to the unmodified sequence, the numbering of unmodified amino acids as they appear in the mutant sequences below remains unchanged following the modification. More than one of the described sequence modifications may be present in an FGF mutant of the present invention. Specifically, the preferred regions for introduction of mutations to create a glycosylation site(s) not present in the wild-type peptide are the nucleotide sequences that encode: amino acids 1-8 (REGION 1; SEQ ID NO:147), amino acids 9-13 (REGION 2; SEQ ID NO:148), amino acids 46-54 (REGION 3; SEQ ID NO:149), amino acids 60-65 (REGION 4; SEQ ID NO:150), amino acids 78-83 (REGION 5; SEQ ID NO:151), amino acids 86-91 (REGION 6; SEQ ID NO:152) or amino acids 112-141 (REGION 7; SEQ ID NO:153), amino acids 149-157 (REGION 8; SEQ ID NO:154), amino acids 160-166 (REGION 9; SEQ ID NO:155), amino acids 167-172 (REGION 10; SEQ ID NO:156), amino acids 173-182 (REGION 11; SEQ ID NO:157) of the wild-type FGF-121 amino acid sequence (see Table 7) can be mutated so that either an N-linked or an O-linked glycosylation site is introduced into the resulting mutated FGF-21 polypeptide.

[0431] The following example describes amino acid sequence mutations introducing N-linked *e.g.,* asparagine residues, and O-linked glycosylation sites, *e.g.*, serine or threonine residues, into a preferably proline-containing site of a wild-type Fibroblast Growth Factor-21 sequence or any modified version thereof.

1. Region 1

[0432] In the Region 1 mutants, the N-terminus of a wild-type FGF-21, M^1HPIPDSS (SEQ ID NO:147), is subdivided into three regions; Region 1(a) M^1HP^3 (SEQ ID NO:158); Region 1(b) M^1HPIP (SEQ ID NO:159); and Region 1(c) P^5DSS (SEQ ID NO:160). Mutations in each region are considered separately, below.

Region 1(a): In these mutants, the wild-type M^1HP^3 (SEQ ID NO:158) is replaced with $M^1X_nB_oO_rJ_qP^3$ wherein B, O, J are independently selected from any uncharged amino acid, and where X is any uncharged amino acid, or histidine (H), and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols n, o, q, r independently represent 0-3. Preferred mutations include:

M^1VTP^3	SEQ ID NO:161
M^1QTP^3;	SEQ ID NO:162

M^1ATP^3;	SEQ ID NO:163
M^1IATP^3;	SEQ ID NO:164

Region 1(b): in these mutants the wild-type M^1HPIP (SEQ ID NO:159) is replaced with $M^1X_nPB_oP$ wherein B is independently selected from any uncharged amino acid, and where X is any uncharged amino acid, or histidine (H), and whereat least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols n, o independently represent 0-3. Preferred mutations include:

M^1FPTP;	SEQ ID NO:165
M^1HPTP;	SEQ ID NO:166
M^1APTP;	SEQ ID NO:167
M^1FPSP;	SEQ ID NO:168
M^1HPSP;	SEQ ID NO:169
M^1APSP;	SEQ ID NO:170
M^1SPTP;	SEQ ID NO:171

Region 1(c): in these mutants the wild-type P^5DSS (SEQ ID NO:160) is replaced with $P^5B_oO_rJ_q$ wherein B, O, J are independently selected from any uncharged amino acid, and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols o, q, r independently represent 0-3. Preferred mutations include:

P^5TSS;	SEQ ID NO:172
P^5TQA;	SEQ ID NO:173
P^5TAQ;	SEQ ID NO:174
P^5TIE;	SEQ ID NO:175
P^5SSS;	SEQ ID NO:176

2. Region 2

[0433] In these mutants, the wild-type P^9L^{10}LQF (SEQ ID NO:148) is replaced with $P^9J_qX_nO_rU_s$ wherein X, J, O, U are independently selected from any uncharged amino acid, and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols q, n, r, s independently represent 0-3. Preferred examples include:

P^9T^{10}TQF;	SEQ ID NO:177
P^9T^{10}INT;	SEQ ID NO:178
P^9T^{10}QGA;	SEQ ID NO:179
P^9T^{10}QGF;	SEQ ID NO:180
P^9T^{10}TVS;	SEQ ID NO:181
P^9T^{10}QAF;	SEQ ID NO:182

3. Region 3

[0434] In these mutants, the wild-type ADQSP^{50}ESLL (SEQ ID NO:149) is replaced with $1_t\emptyset Z_mB_oP^{50}J_qX_nO_rU_s$ wherein Ø, Z, X, B, J, O, U, 1, 2, 3 are independently selected from any uncharged amino acid, where Z or J is independently selected as glutamic acid (E), where 2 and X may be independently selected as lysine (K) or arginine (R), and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols t, m, n, o, q, r, s, t independently represent 0-3. Preferred examples include:

ADQSP^{50}TSLL;	SEQ ID NO:183
ADQSP^{50}TTVS;	SEQ ID NO:184
ADQSP^{50}TINT;	SEQ ID NO:185
ADQSP^{50}TQAL;	SEQ ID NO:186
ADQSP^{50}TQGA;	SEQ ID NO:187
ADQSP^{50}TQAL;	SEQ ID NO:188
ATQSP^{50}ESLL;	SEQ ID NO:189
ATESP^{50}ESLL;	SEQ ID NO:190

ATETP^{50}ESLL;	SEQ ID NO:191
VTQSP^{50}ESLL;	SEQ ID NO:192
VTETP^{50}ESLL;	SEQ ID NO:193
ATESP^{50}ASLL;	SEQ ID NO:194

4. Region 4

[0435] In these mutants, the wild-type KP^{61}GVIQ (SEQ ID NO:150) is replaced with $B_oP^{61}J_qX_nO_rU_s$ wherein B is selected from lysine (K) or any uncharged amino acid, where X, J, O, U are independently selected from any uncharged amino acid, and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols n, o, q, r, s independently represent 0-3. Preferred examples include:

SP^{61}TVIQ;	SEQ ID NO:195
AP^{61}TVIQ;	SEQ ID NO:196
SP^{61}TTVS;	SEQ ID NO:197
SP^{61}TINT;	SEQ ID NO:198
SP^{61}TQAQ;	SEQ ID NO:199
SP^{61}TQGA;	SEQ ID NO:200
SP^{61}TVIA;	SEQ ID NO:201
AP^{61}TTVS;	SEQ ID NO:202
AP^{61}TINT;	SEQ ID NO:203

5. Region 5

[0436] In these mutants, the wild-type RP^{79}DGAL (SEQ ID NO:151) is replaced with $B_oP^{79}J_qX_nO_rU_s$ wherein X, B, J, O, U are independently selected from any uncharged amino acid, where B may be independently selected as lysine (K) or arginine (R), and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols n, o, q, r, s independently represent 0-3. Preferred examples include:

$SP^{79}TGAL$;	SEQ ID NO:204
$AP^{79}TGAL$;	SEQ ID NO:205
$SP^{79}TINT$;	SEQ ID NO:206
$SP^{79}TTVS$;	SEQ ID NO:207
$SP^{79}TQAL$;	SEQ ID NO:208
$AP^{79}TQAL$;	SEQ ID NO:209
$SP^{79}TQGA$;	SEQ ID NO:210
$SP^{79}TQGAM$;	SEQ ID NO:211

6. Region 6

[0437] In these mutants, the wild-type $SLHFDP^{91}$ (SEQ ID NO:152) is replaced with $21_t\text{ø}Z_mB_oP^{91}$ wherein Ø, Z, B, 1, 2 are independently selected from any uncharged amino acid, and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols t, m, o independently represent 0-3. Preferred examples include:

$SLTFTP^{91}$;	SEQ ID NO:212
$SLTETP^{91}$;	SEQ ID NO:213
$SVTETP^{91}$;	SEQ ID NO:214

7. Region 7

[0438] In these mutants, the wild-type A^{112}HGLPLHLPGNKSPHRDPAPRGPARFLPLP (SEQ ID NO:153) is subdivided into five regions; Region 7(a) $AHGLP^{116}LHLP^{120}$ (SEQ ID NO:215); Region 7(b) $HLP^{120}GNKSP^{125}HR$ (SEQ ID NO:216); Region 7(c) $KSP^{125}HRDP^{129}APR$ (SEQ ID NO:217); Region 7(d) $RGP^{134}ARFLP^{139}LP$ (SEQ ID NO:218); and Region 7(e) $RGP^{134}ARFLP^{139}LP$ (SEQ ID NO:219). Mutations in each region are considered separately, below.

Region 7(a): In these mutants, the wild-type $AHGLP^{116}LHLP^{120}$ (SEQ ID NO:215) is replaced with $1_t\text{ø}Z_mB_oP^{116}J_qX_nO_rP^{120}$ wherein 1, Ø, Z, X, B, J, O are independently selected from any uncharged amino acid, and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols t, m, o, q, n, r independently represent 0-3. Preferred mutations include:

ATGTP^{116}LHLP^{120};	SEQ ID NO:220
ATETP^{116}LHLP^{120};	SEQ ID NO:221
VTETP^{116}LHLP^{120};	SEQ ID NO:222
VTGLP^{116}LHLP^{120};	SEQ ID NO:223
ATGLP^{116}LHLP^{120};	SEQ ID NO:224
AHGLP^{116}TQAP^{120};	SEQ ID NO:225
AHGLP^{116}TAQP^{120};	SEQ ID NO:226
AHGLP^{116}TEIP^{120};	SEQ ID NO:227
AHGLP^{116}TSSP^{120};	SEQ ID NO:228
AHGLP^{116}TALP^{120};	SEQ ID NO:229
ASGLP^{116}TQAP^{120};	SEQ ID NO:230
ASGLP^{116}TEIP^{120};	SEQ ID NO:231

Region 7(b): In these mutants, the wild-type HLP^{120}GNKSP^{125}HR (SEQ ID NO:216) is replaced with $1_tLP^{120}X_nO_rU_s2_aP^{125}B_oJ_q$ wherein X, B, J, O, U, 1, 2 are independently selected from any uncharged amino acid, where B, J, 1 are independently selected as histidine (H), lysine (K), or arginine (R), and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols t, n, r, s, a, o, q independently represent 0-3. Preferred mutations include:

$HLP^{120}TTAVP^{125}HR$;	SEQ ID NO:232
$HLP^{120}TSGEP^{125}HR$;	SEQ ID NO:233
$HLP^{120}GSTAP^{125}HR$;	SEQ ID NO:234
$HLP^{120}GNTSP^{125}HR$;	SEQ ID NO:235
$HLP^{120}GTESP^{125}HR$;	SEQ ID NO:236
$HLP^{120}LTQTP^{125}HR$;	SEQ ID NO:237
$HLP^{120}GTQTP^{125}HR$;	SEQ ID NO:238
$HLP^{120}LTQTP^{125}AR$;	SEQ ID NO:239
$HLP^{120}TNASP^{125}HR$;	SEQ ID NO:240
$HLP^{120}TQGSP^{125}HR$;	SEQ ID NO:241
$HLP^{120}VTSQP^{125}HR$;	SEQ ID NO:242
$HLP^{120}TINTP^{125}HR$;	SEQ ID NO:243
$HLP^{120}TSVSP^{125}HR$;	SEQ ID NO:244

Region 7(c): In these mutants, the wild-type $KSP^{125}HRDP^{129}APR$ (SEQ ID NO:217) is replaced with $1_tSP^{125}X_nO_rU_sP^{129}B_oPJ_q$ wherein B, U, 1 are independently selected from any uncharged amino acid, where X, O, J are independently selected from any uncharged amino acid or histine (H), lysine (K), or arginine (R), and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols t, n, r, s, o, q independently represent 0-3. Preferred mutations include:

$KSP^{125}TAQP^{129}APR$;	SEQ ID NO:245
$KSP^{125}TADP^{129}APR$;	SEQ ID NO:246
$ASP^{125}TAQP^{129}APR$;	SEQ ID NO:247
$SSP^{125}TADP^{129}APR$;	SEQ ID NO:248
$KSP^{125}TSDP^{129}APR$;	SEQ ID NO:249
$KSP^{125}TEIP^{129}APR$;	SEQ ID NO:250
$KSP^{125}TEIP^{129}APR$;	SEQ ID NO:251
$KSP^{125}TEDP^{129}APR$;	SEQ ID NO:252
$ASP^{125}TEDP^{129}APR$;	SEQ ID NO:253
$SSP^{125}TADP^{129}APR$;	SEQ ID NO:254
$SSP^{125}TAQP^{129}APR$;	SEQ ID NO:255
$KSP^{125}TQAP^{129}APR$;	SEQ ID NO:256
$SSP^{125}TQAP^{129}APR$;	SEQ ID NO:257
$ASP^{125}TEIP^{129}APR$;	SEQ ID NO:258

$KSP^{125}HRDP^{129}TPR$; SEQ ID NO:259
$KSP^{125}HRDP^{129}SPR$; SEQ ID NO:260
$KSP^{125}HRDP^{129}TPA$; SEQ ID NO:261
$KSP^{125}HRDP^{129}TPS$; SEQ ID NO:262
$KSP^{125}HSDP^{129}TPA$; SEQ ID NO:263
$KSP^{125}HADP^{129}TPS$; SEQ ID NO:264
$KSP^{125}HADP^{129}TPA$; SEQ ID NO:265

Region 7(d): In these mutants, the wild-type $RGP^{134}ARFLP^{139}LP$ (SEQ ID NO:218) is replaced with $1_tGP^{134}X_nO_rU_s2_aP^{139}B_oP$ wherein X, B, O, U, 1, 2 are independently selected from any uncharged amino acid, where O, 1 are independently selected from lysine (K) or arginine (R), and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols t, n, r, s, a, o independently represent 0-3. Preferred mutations include:

$RGP^{134}TSFLP^{139}LP$; SEQ ID NO:266
$RGP^{134}TSGEP^{139}LP$; SEQ ID NO:267
$RGP^{134}GSTAP^{139}LP$; SEQ ID NO:268
$RGP^{134}ANTSP^{139}LP$; SEQ ID NO:269
$RGP^{134}ATESP^{139}LP$; SEQ ID NO:270
$RGP^{134}ATQTP^{139}LP$; SEQ ID NO:271
$RGP^{134}ATQTP^{139}LP$; SEQ ID NO:272
$RGP^{134}LTQTP^{139}LP$; SEQ ID NO:273
$RGP^{134}TQFLP^{139}LP$; SEQ ID NO:274
$RGP^{134}TSFLP^{139}LP$; SEQ ID NO:275
$RGP^{134}VTSQP^{139}LP$; SEQ ID NO:276
$SGP^{134}TSFLP^{139}LP$; SEQ ID NO:277
$AGP^{134}TSGEP^{139}LP$; SEQ ID NO:278
$SGP^{134}TSALP^{139}LP$; SEQ ID NO:279

Region 7(e): In these mutants, the wild-type $RGP^{134}ARFLP^{139}LP$ (SEQ ID NO:219) is replaced with $1_tGP^{134}X_nO_rU_s2_aP^{139}B_oP$ wherein X, B, O, U, 1, 2 are independently selected from any uncharged amino acid, where O, 1 are independently selected from lysine (K) or arginine (R), and where at least one is T or S, and is a substrate for GalNAc transferase where

GalNAc is added to at least T or S. The symbols t, n, r, s, a, o independently represent 0-3. Preferred mutations include:

$RGP^{134}ARFLP^{139}TP$;	SEQ ID NO:280
$RGP^{134}ARFLP^{139}SP$;	SEQ ID NO:281
$RGP^{134}ASFLP^{139}TP$;	SEQ ID NO:282

8. Region 8

[0439] In these mutants, the wild-type $EPP^{151}GILAP^{156}Q$ (SEQ ID NO:154) is replaced with $B_OPP^{151}X_nO_rU_s2_aP^{156}1_t$ wherein B, X, O, U, 2, 1 are independently selected from any uncharged amino acid, and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols o, n, r, s, a, t independently represent 0-3. Preferred mutations include:

$TPP^{151}GILAP^{156}Q$;	SEQ ID NO:283
$SPP^{151}GILAP^{156}Q$;	SEQ ID NO:284
$EPP^{151}TILAP^{156}Q$;	SEQ ID NO:285
$EPP^{151}TTLAP^{156}Q$;	SEQ ID NO:286
$EPP^{151}TQLAP^{156}Q$;	SEQ ID NO:287
$EPP^{151}TQGAP^{156}Q$;	SEQ ID NO:288
$EPP^{151}TSGEP^{156}Q$;	SEQ ID NO:289
$EPP^{151}GSTAP^{156}Q$;	SEQ ID NO:290
$EPP^{151}TTAVP^{156}Q$;	SEQ ID NO:291
$EPP^{151}GNTSP^{156}Q$;	SEQ ID NO:292
$EPP^{151}GTESP^{156}Q$;	SEQ ID NO:293
$EPP^{151}GTETP^{156}Q$;	SEQ ID NO:294
$EPP^{151}VTSQP^{156}Q$;	SEQ ID NO:295
$EPP^{151}AVQTP^{156}Q$;	SEQ ID NO:296
$EPP^{151}LTQTP^{156}Q$;	SEQ ID NO:297
$EPP^{151}VTSQP^{156}Q$;	SEQ ID NO:298
$EPP^{151}SSGAP^{156}Q$;	SEQ ID NO:299
$EPP^{151}TINTP^{156}Q$;	SEQ ID NO:300
$EPP^{151}TTVSP^{156}Q$;	SEQ ID NO:301
$EPP^{151}TQAAP^{156}Q$;	SEQ ID NO:302
$EPP^{151}GILAP^{156}T$;	SEQ ID NO:303
$EPP^{151}GILAP^{156}S$;	SEQ ID NO:304

9. Region 9

[0440] In these mutants, the wild-type DVGSSDP^{166} (SEQ ID NO:155) is replaced with $X_nO_rU_s2_aB_OZ_mP^{166}$ wherein Z, X, B, O, U, 2 are independently selected from any uncharged amino acid, glutamic acid (E), or aspartic acid (D), and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols n, r, s, a, o, m independently represent 0-3. Preferred mutations include:

TVGSSDP^{166};	SEQ ID NO:305
DVGSSTP^{166};	SEQ ID NO:306
DVGTETP^{166};	SEQ ID NO:307
DAASAAP^{166};	SEQ ID NO:308
DAATAAP^{166};	SEQ ID NO:309
DVGTSDP^{166};	SEQ ID NO:310
DVATSDP^{166};	SEQ ID NO:311
TGDSSDP^{166};	SEQ ID NO:312
TDASGAP^{166};	SEQ ID NO:313
DVGTSGP^{166};	SEQ ID NO:314

10. Region 10

[0441] In these mutants, the wild-type LSMVGP^{172} (SEQ ID NO:156) is replaced with $X_nO_rU_s2_aB_oP^{172}$ wherein B, O, U, 2 are independently selected from any uncharged amino acid, glutamic acid (E), or aspartic acid (D), where at least X is selected as either T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols n, r, s, a, o independently represent 0-3. Preferred mutations include:

TSMVGP;	SEQ ID NO:315
TSGVGP;	SEQ ID NO:316
TSGAMP;	SEQ ID NO:317
TQGAMP;	SEQ ID NO:318
TSMVGP;	SEQ ID NO:319
TQGAMP;	SEQ ID NO:320

11. Region 11

[0442] In these mutants, the wild-type SQGRSP^{178}SYAS (SEQ ID NO:157) is subdivided into two regions; Region 11(a) SQGRSP^{178} (SEQ ID NO:321); and the carboxy terminal

Region 11(b) $RSP^{178}SYAS$ (SEQ ID NO:322). Mutations in each region are considered separately, below.

Region 11(a): In these mutants, the wild-type $SQGRSP^{178}$ (SEQ ID NO:321) is replaced with $X_nO_rU_s2_aB_oP^{178}$ wherein B, O, U, 2 are independently selected from any uncharged amino acid, glutamic acid (E), or aspartic acid (D), where at least X is selected as either T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols n, r, s, a, o independently represent 0-3. Preferred mutations include:

$SQGASP^{178}$;	SEQ ID NO: 323
$TQGASP^{178}$;	SEQ ID NO: 324
$TQGAMP^{178}$;	SEQ ID NO: 325
$TQGAMp^{178}$;	SEQ ID NO: 326

Region 11(b): In these mutants, the wild-type carboxy terminal $RSP^{178}SYAS$ (SEQ ID NO:322) is replaced with $ZSP^{178}X_nO_rU_s1B_o23$ wherein Z, X, B, O, U, 1, 2, 3 are independently selected from any uncharged amino acid, glutamic acid (E), or aspartic acid (D), where at least X is selected as either T or S, where Z may be independently selected as arginine (R) or lysine (K), and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols n, r, s, o independently represent 0-3. Preferred mutations include:

$ASP^{178}SYAS$;	SEQ ID NO: 327
$RSP^{178}TSAVAA$;	SEQ ID NO: 328
$ASP^{178}TSAVAA$;	SEQ ID NO: 329
$ASP^{178}SSGAPPPS$;	SEQ ID NO: 330
$ASP^{178}SSGAPP$;	SEQ ID NO: 331
$ASP^{178}SSGAP$;	SEQ ID NO: 332
$RSP^{178}SSGAPPPS$;	SEQ ID NO: 333
$ASP^{178}TINT$;	SEQ ID NO: 334
$ASP^{178}TSVS$;	SEQ ID NO: 335
$ASP^{178}TQAF$;	SEQ ID NO: 336
$ASP^{178}TINTP$;	SEQ ID NO: 337

EXAMPLE 4

Soluble Expression of FGF-20 and FGF-21 in *E. coli*

[0443] Therapeutic proteins are commonly expressed in *E. coli* as inactive, insoluble inclusion bodies. Following inclusion body purification, soluble therapeutics are obtained by a protein refolding reaction. This refolding process is typically enhanced by the inclusion of compounds that facilitate the reshuffling of disulfide bonds.

[0444] The *E. coli* cytoplasm, the site of the protein expression and inclusion body formation, is a chemically reducing environment that inhibits the formation of disulfide bonds. A strain that has a less reducing, more oxidizing cytoplasm would theoretically permit disulfide bond formation, facilitating the expression of therapeutic proteins in a soluble form.

Experimental:

[0445] Therapeutic proteins tested were human FGF-20 and FGF-21. The FGF-21 construct lacked its N-terminal signal sequence. Genes encoding these therapeutic proteins were cloned into up to four different vector backbones (Vector #1, Vector #2, Vector #3, and pET24a) as indicated in Table 8. These constructs were tested in one or two of four different bacterial strains (W3110, BL21 DE3, E. $coli_{(trxb,gor,supp)}$-2, and E. $coli_{(trxb,gor,supp)}$-2 DE3) as indicated in Table 8.

[0446] For protein expression, an overnight small scale culture was used to inoculate a 100 mL culture of prewarmed martone LB containing 50 μg/mL kanamycin. The culture was

incubated at 37°C with shaking, and monitored for OD_{620}. When the OD_{620} reached 0.4-0.6, the cultures were split and transferred to a 37°C or 20°C shaking incubator for 15-20 minutes. IPTG was then added to 0.1 – 1.0 mM final concentration, and shaking incubation was continued for 1.5 hours up to overnight. Cells were harvested by centrifugation at 4°C, 7000xg for 15 mins in a Sorvall RC3C+.

[0447] For whole cell extract analysis of protein expression, cells from a 150 µL aliquot of the induced cultures were collected by centrifugation and lysed in 1xPBS/0.1% SDS. Following heating with 100 mM DTT and 1x protein sample buffer, samples were resolved by SDS-PAGE, and stained with Coomassie fluorescent orange.

[0448] For the analysis of protein solubility, bacterial cell pellets from 50-100 mL of induced cultures were resuspended using ~30 mL of lysis buffer (eg 1x PBS, 5mM EDTA), and lysed by mechanical disruption with three passes through a microfluidizer. Small samples were taken and insoluble material was pelleted by centrifugation for 10 minutes at top speed at 4°C in a microcentrifuge. Following the spin, the supernatant was separated from the pellet, and both were analyzed by SDS-PAGE and protein staining. Western blot analysis with antibodies specific for the therapeutic proteins was also carried out to verify the identity of the observed soluble proteins.

Results:

FGF-20

[0449] Vectors bearing FGF-20 were transformed into different bacterial strains as indicated in Table 8. 50-100 mL induction cultures, varying by temperature, aeration (rpm), IPTG concentration, and time, were analyzed by whole cell extract (WCE) SDS-PAGE. As shown in **FIG. 1a**, moderate expression was observed in Vector #2, Vector #3, and pET24a vectors, but not in Vector #1. Expression was observed as soon as 1.5 hours after induction, and greater levels of expression were at 37°C than 20°C.

[0450] To determine whether FGF-20 was expressed as a soluble protein, induced cell pellets of BL21 DE3 and E. $coli_{(trxb,gor,supp)}$-2 DE3 strains bearing pET24a FGF-20 were lysed, centrifuged, and analyzed by SDS-PAGE. As shown in **FIG. 1b**, the majority of FGF-20 was soluble in the E. $coli_{(trxb,gor,supp)}$-2 DE3 cells when grown at 20°C. Growth at 37°C yielded approximately equal amount of soluble and insoluble protein in both BL21 DE3 and E. $coli_{(trxb,gor,supp)}$-2 DE3 cells.

FGF-21

[0451] Vectors bearing FGF-21 were transformed into different bacterial strains as indicated in Table 8. 100 mL induction cultures, varying by temperature and time, were analyzed by WCE SDS-PAGE. As shown in **FIG. 1c**, expression was observed in Vector #2, Vector #3, and pET24a vectors, but not in Vector #1. Expression was observed as soon as 1.5 hours after induction. As rFGF-21 was observed to resolve by SDS-PAGE approximately 3-5 kDa larger than the expected ~19.7 kDa, the identity of the induced band was confirmed by Western blot (**FIG. 1c**).

[0452] To determine whether FGF-21 was expressed as a soluble protein, induced cell pellets of W3110 and E. coli$_{(trxb,gor,supp)}$ 2 strains bearing Vector #3 FGF-21 were lysed, centrifuged, and analyzed by SDS-PAGE. As shown in **FIG. 1d**, the majority of FGF-21 was soluble only in the E. coli$_{(trxb,gor,supp)}$ 2 cells when induced at 20°C. Induction in E. coli$_{(trxb,gor,supp)}$-2 cells at 37°C, or in W3110 cells at either temperature yielded predominantly insoluble protein.

[0453] This study demonstrated a method for expressing the therapeutic proteins FGF-20 and FGF-21 in bacteria as soluble proteins. The expression technique using E. coli$_{(trxb,gor,supp)}$-2 described here should be applicable for the soluble expression of other therapeutic proteins.

[0454] While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention.

[0455] All patents, patent applications, and other publications cited in this application are incorporated by reference in their entirety for all purposes.

WHAT IS CLAIMED IS:

1. A Fibroblast Growth Factor (FGF) conjugate comprising:

a FGF peptide; and

a modifying group, wherein said modifying group is covalently attached to said peptide at a preselected glycosyl or amino acid residue of said peptide via an intact glycosyl linking group.

2. The FGF conjugate of claim **1**, wherein said FGF peptide is a member selected from FGF-1, FGF-2, FGF-18, FGF-20 and FGF-21.

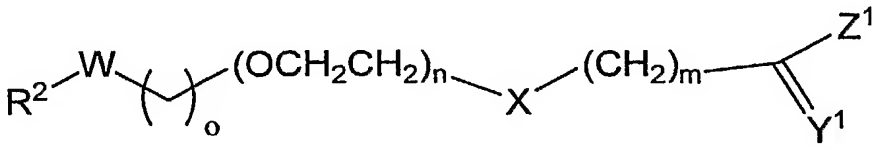
3. The FGF conjugate of claim **1**, wherein said FGF peptide is at least 95% homologous to at least one amino acid sequence selected from the group consisting of SEQ ID NOs: 1, 9-14, 18-45, 48-65, 69-109, 112-146, 161-214, 220-320 and 323-360.

4. The FGF conjugate of claim **1**, wherein said modifying group is covalently attached at said preselected glycosyl residue.

5. The FGF conjugate of claim **4**, wherein said modifying group is a non-glycosidic modifying group.

6. The FGF conjugate of claim **5**, wherein said non-glycosidic modifying group is a member selected from linear PEG and branched PEG.

7. The FGF conjugate of claim **6**, wherein said PEG moiety is linear PEG and said linear PEG has a structure according to the following formula:



in which

R^2 is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted heteroalkyl, *e.g.*, acetal, OHC-, H_2N-CH_2CH_2-, HS-CH_2CH_2-, and-$(CH_2)_qC(Y^1)Z^2$; -sugar-nucleotide, or protein;

n is an integer selected from 1 to 2500;

m, o, and q are integers independently selected from 0 to 20;

Z is a member selected from OH, NH_2, halogen, S-R^3, the alcohol portion of activated esters, -$(CH_2)_pC(Y^2)V$, -$(CH_2)_pU(CH_2)_sC(Y^2)_v$, sugar-nucleotide, protein, and leaving groups, *e.g.*, imidazole, p-nitrophenyl, HOBT, tetrazole, halide;

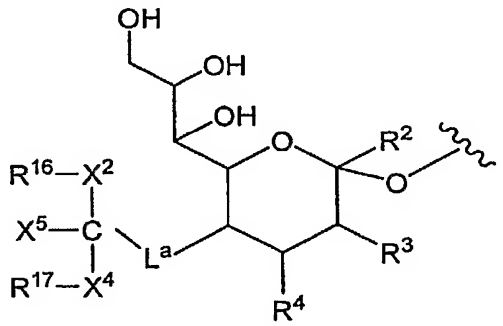
X, Y^1, Y^2, W and U are independently selected from O, S, N-R^4;

V is a member selected from OH, NH_2, halogen, S-R^5, the alcohol component of activated esters, the amine component of activated amides, sugar-nucleotides, and proteins;

p, s and v are integers independently selected from 0 to 20; and

R^3, R^4 and R^5 are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heterocycloalkyl and substituted or unsubstituted heteroaryl.

8. The FGF conjugate of claim **1**, wherein said glycosyl linking group has a structure according to the following formula:



wherein

R^2 is H, CH_2OR^7, $COOR^7$ or OR^7

wherein

R^7 represents H, substituted or unsubstituted alkyl or substituted or unsubstituted heteroalkyl;

R^3 and R^4 are members independently selected from H, substituted or unsubstituted alkyl, OR^8, $NHC(O)R^9$

wherein

R^8 and R^9 are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl or sialic acid;

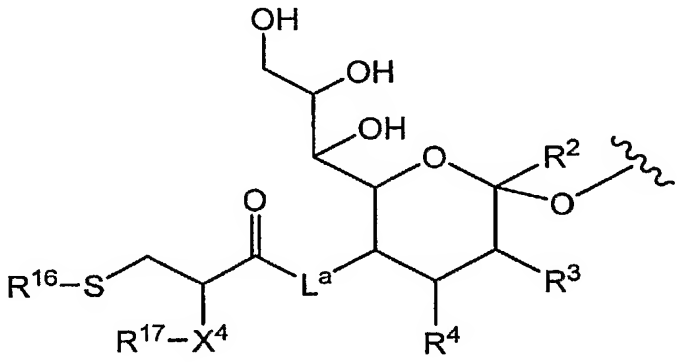
L^a is a linker selected from a bond, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl

R^{16} and R^{17} are independently selected polymeric arms;

X^2 and X^4 are independently selected linkage fragments joining polymeric moieties R^{16} and R^{17} to C; and

X^5 is a non-reactive group.

9. The FGF conjugate of claim **8**, wherein said glycosyl linking group has a structure according to the following formula:



10. An isolated nucleic acid comprising a polynucleotide sequence encoding a mutant Fibroblast Growth Factor, wherein the mutant Fibroblast Growth Factor comprises a newly introduced N-linked or O-linked glycosylation site that does not exist in the corresponding wild-type Fibroblast Growth Factor.

11. The nucleic acid of claim **10**, wherein the nucleic acid encodes a mutant Fibroblast Growth Factor-20 that has a corresponding wild-type Fibroblast Growth Factor-20 amino acid sequence that is at least 95% homologous to SEQ ID NO:1.

12. The nucleic acid of claim **10**, wherein the newly introduced glycosylation site is near a proline residue.

13. The nucleic acid of claim **12**, wherein the proline residue is located at position 3, 28, 29, 34, 35, 52, 81, 175, 192, 197, or 201 of SEQ ID NO:1.

14. The nucleic acid of claim **10**, wherein the mutant Fibroblast Growth Factor is at least 95% homologous to at least one amino acid sequence selected from the

group consisting of SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, 112-145, 161-214, 220-320 and 323-360.

15. The nucleic acid of claim **10**, wherein the mutant Fibroblast Growth Factor comprises more than one newly introduced glycosylation site.

16. An expression cassette comprising the nucleic acid of claim **10**.

17. A cell comprising the nucleic acid of claim **10**.

18. A mutant Fibroblast Growth Factor, comprising a newly introduced N-linked or O-linked glycosylation site that does not exist in the corresponding wild-type Fibroblast Growth Factor.

19. The mutant Fibroblast Growth Factor of claim **18**, wherein the corresponding wild-type Fibroblast Growth Factor has the amino acid sequence that is at least 95% homologous to SEQ ID NO:1.

20. The mutant Fibroblast Growth Factor of claim **18**, wherein the newly introduced glycosylation site is proximate to a proline residue.

21. The mutant Fibroblast Growth Factor of claim **20**, wherein the proline residue is located at position 3, 28, 29, 34, 35, 52, 81, 175, 192, 197, or 201 of SEQ ID NO:1.

22. The mutant Fibroblast Growth Factor of claim **18**, wherein said mutant FGF is at least 95% homologous to at least one amino acid sequence selected from the group consisting of SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, 112-145, 161-214, 220-320 and 323-360.

23. The mutant Fibroblast Growth Factor of claim **18**, wherein the mutant Fibroblast Growth Factor comprises more than one newly introduced glycosylation site.

24. The mutant Fibroblast Growth Factor of claim **18**, comprising a water-soluble polymer attached to a glycosylation site through a glycosyl linker.

25. The mutant Fibroblast Growth Factor of claim **24**, wherein said glycosyl linker is an intact glycosyl linker.

26. A method for making a mutant Fibroblast Growth Factor, which comprises a newly introduced N-linked or O-linked glycosylation that does not exist in the corresponding wild-type Fibroblast Growth Factor, comprising the steps of:

(a) recombinantly producing the mutant Fibroblast Growth Factor; and

(b) glycosylating the mutant Fibroblast Growth Factor at the newly introduced glycosylation site

wherein

said glycosylating is a cell free, in vitro process .

27. The method of claim **26**, wherein the corresponding wild-type Fibroblast Growth Factor has the amino acid sequence that is at least 95% homologous to SEQ ID NO:1.

28. The method of claim **26**, wherein the newly introduced glycosylation site is near a proline residue.

29. The method of claim **28**, wherein the proline residue is located at position 3, 28, 29, 34, 35, 52, 81, 175, 192, 197, or 201 of SEQ ID NO:1.

30. The method of claim **26**, wherein the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from the group consisting of SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, 112-145, 161-214, 220-320 and 323-360.

31. The method of claim **26**, wherein the mutant Fibroblast Growth Factor comprises more than one newly introduced glycosylation site.

32. A pharmaceutical composition comprising an effective amount of a mutant Fibroblast Growth Factor, which comprises a newly introduced N-linked or O-linked glycosylation that does not exist in the corresponding wild-type Fibroblast Growth Factor.

33. The composition of claim **32**, wherein the corresponding wild-type Fibroblast Growth Factor has the amino acid sequence that is at least 95% homologous to SEQ ID NO:1.

34. The composition of claim **32**, wherein the newly introduced glycosylation site is near a proline residue.

35. The composition of claim 34, wherein the proline residue is located at position 3, 28, 29, 34, 35, 52, 81, 175, 192, 197, or 201 of SEQ ID NO:1.

36. The composition of claim 32, wherein the mutant Fibroblast Growth Factor is at least 95% homologous to at least one amino acid sequence selected from the group consisting of SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, 112-145, 161-214, 220-320 and 323-360.

37. The composition of claim 32, wherein the mutant Fibroblast Growth Factor comprises more than one newly introduced glycosylation site.

38. A method for treating Fibroblast Growth Factor deficiency in a patient, comprising the step of administering an effective amount of a mutant Fibroblast Growth Factor to the patient, wherein the mutant Fibroblast Growth Factor comprises a newly introduced N-linked or O-linked glycosylation that does not exist in the corresponding wild-type Fibroblast Growth Factor.

39. The method of claim 38, wherein the corresponding wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:1.

40. The method of claim 38, wherein the newly introduced glycosylation site is near a proline residue.

41. The method of claim 40, wherein the proline residue is located at position 3, 28, 29, 34, 35, 52, 81, 175, 192, 197, or 201 of SEQ ID NO:1.

42. The method of claim 38, wherein the mutant Fibroblast Growth Factor is at least 95% homologous to at least one amino acid sequence selected from the group consisting of SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, 112-145, 161-214, 220-320 and 323-360.

43. The method of claim 38, wherein the mutant Fibroblast Growth Factor comprises more than one newly introduced glycosylation site.

44. A method for making a glycoconjugate of a mutant Fibroblast Growth Factor, which comprises a newly introduced N-linked or O-linked glycosylation that does not exist in the corresponding wild-type Fibroblast Growth Factor, comprising the steps of:

(a) recombinantly producing the mutant Fibroblast Growth Factor, and

(b) enzymatically glycosylating the mutant Fibroblast Growth Factor with a modified sugar at the newly introduced glycosylation site

wherein

said glycosylating is a cell free, in vitro process.

45. The method of claim **44**, wherein the modified sugar is modified with a water-soluble polymer.

46. The method of claim **45**, wherein the modified sugar is modified with a water soluble polymer selected from the group consisting of poly(ethylene glycol) and m-poly(ethylene glycol).

47. The method of claim **44** wherein the corresponding wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:1.

48. The method of claim **45**, wherein the newly introduced glycosylation site is near a proline residue.

49. The method of claim **44**, wherein the proline residue is located at position 3, 28, 29, 34, 35, 52, 81, 175, 192, 197, or 201 of SEQ ID NO:1.

50. The method of claim **44**, wherein the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from the group consisting of SEQ ID NOs: SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, 112-145, 161-214, 220-320 and 323-360.

51. The method of claim **44**, wherein the mutant Fibroblast Growth Factor comprises more than one newly introduced glycosylation site.

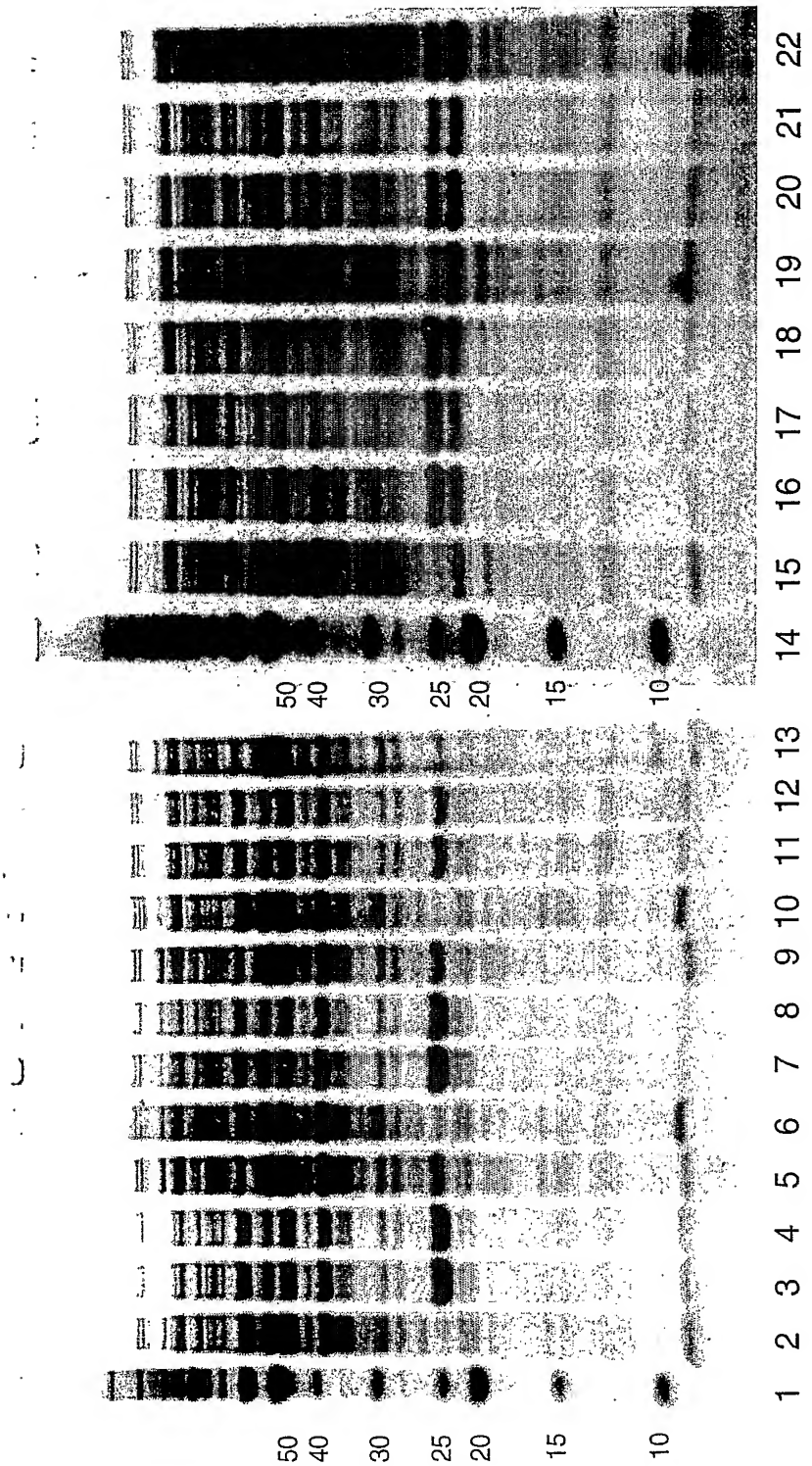

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
50 40 30 25 20 15 10
50 40 30 25 20 15 10

FIG. 1A

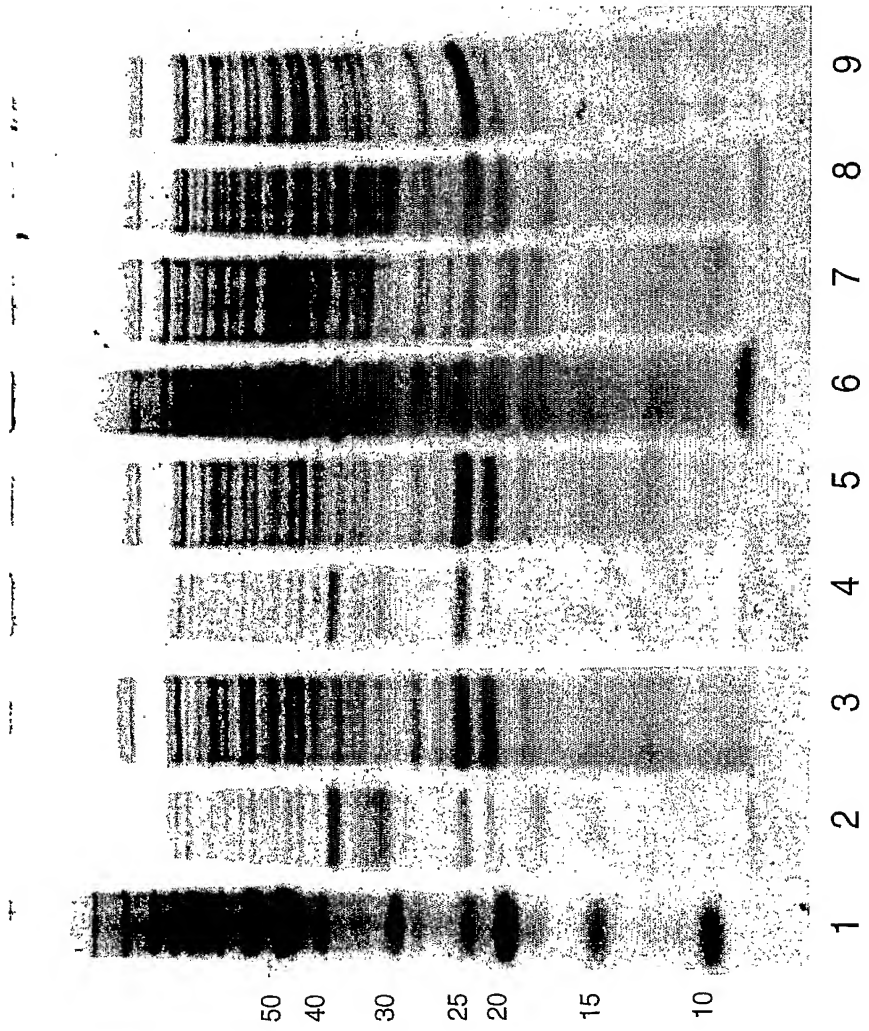


FIG. 1B

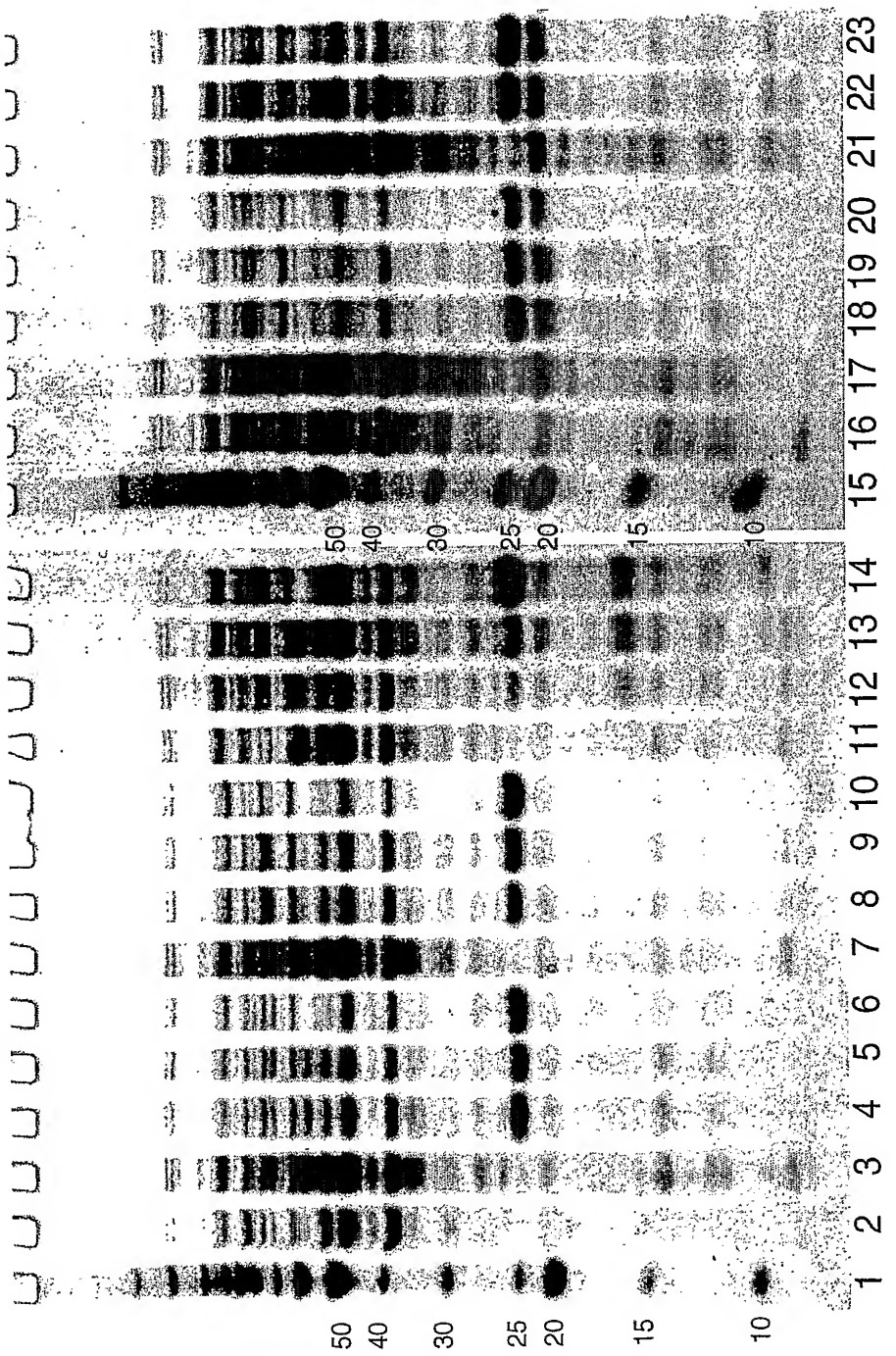

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
50 40 30 25 20 15 10

FIG. 1C

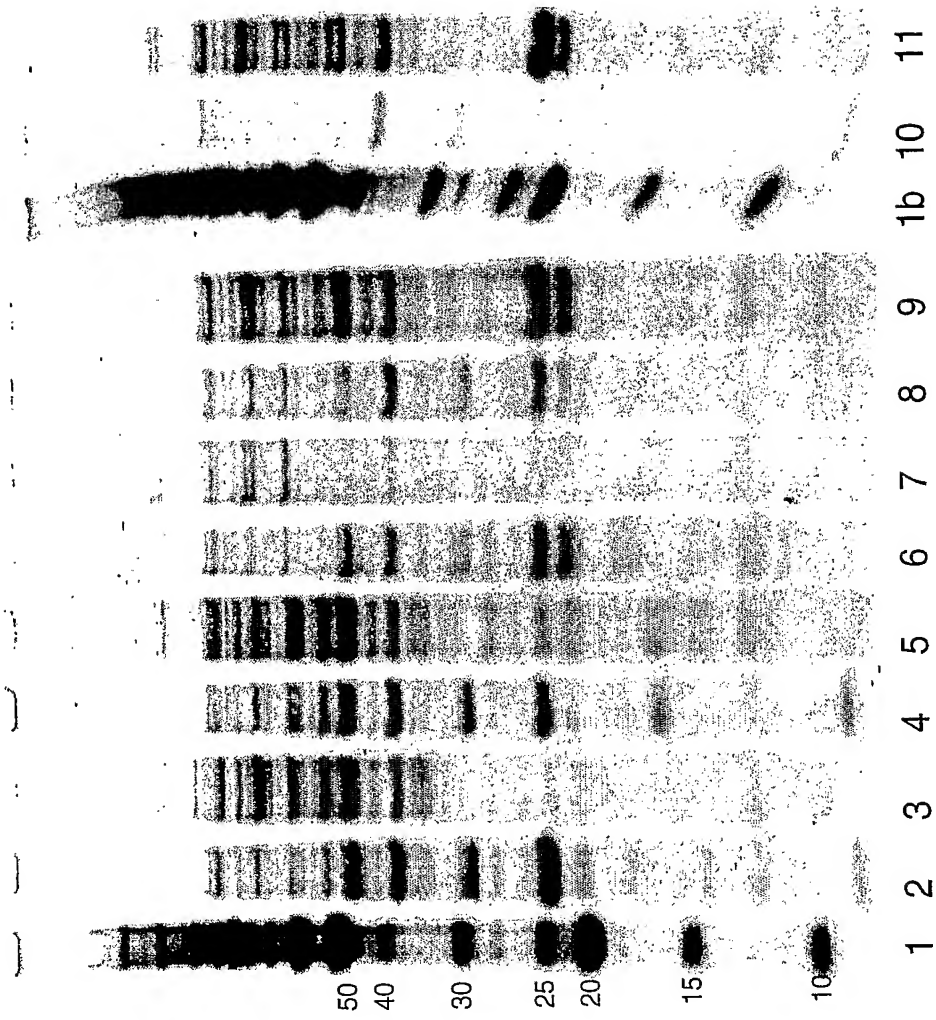


FIG. 1D

FIG. 2A

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB /3D
At1g08280	*Arabidopsis thaliana*	n.d.	AC011438 BT004583 NC_003070	AAF18241.1 AAO42829.1 NP_172305.1	Q84W00 Q9SGD2	
At1g08660/F22O13.14	*Arabidopsis thaliana*	n.d.	AC003981 AY064135 AY124807 NC_003070 NM_180609	AAF99778.1 AAL36042.1 AAM70516.1 NP_172342.1 NP_850940.1	Q8VZJ0 Q9FRR9	
At3g48820/T21J18_90	*Arabidopsis thaliana*	n.d.	AY080589 AY133816 AL132963 NM_114741	AAL85966.1 AAM91750.1 CAB87910.1 NP_190451.1	Q8RY00 Q9M301	
α-2,3-sialyltransferase (ST3GAL-IV)	*Bos taurus*	n.d.	AJ584673	CAE48298.1		
α-2,3-sialyltransferase (St3Gal-V)	*Bos taurus*	n.d.	AJ585768	CAE51392.1		
α-2,6-sialyltransferase (Siat7b)	*Bos taurus*	n.d.	AJ620651	CAF05850.1		
α-2,8-sialyltransferase (SIAT8A)	*Bos taurus*	2.4.99.8	AJ699418	CAG27880.1		
α-2,8-sialyltransferase (Siat8D)	*Bos taurus*	n.d.	AJ699421	CAG27883.1		
α-2,8-sialyltransferase ST8Siα-III (Siat8C)	*Bos taurus*	n.d.	AJ704563	CAG28696.1		
CMP α-2,6-sialyltransferase (ST6Gal I)	*Bos taurus*	2.4.99.1	Y15111 NM_177517	CAA75385.1 NP_803483.1	O18974	
sialyltransferase 8 (fragment)	*Bos taurus*	n.d.	AF450088	AAL47018.1	Q8WN13	
sialyltransferase ST3Gal-II (Siat4B)	*Bos taurus*	n.d.	AJ748841	CAG44450.1		
sialyltransferase ST3Gal-III (Siat6)	*Bos taurus*	n.d.	AJ748842	CAG44451.1		
sialyltransferase ST3Gal-VI (Siat10)	*Bos taurus*	n.d.	AJ748843	CAG44452.1		
ST3Gal I	*Bos taurus*	n.d.	AJ305086	CAC24698.1	Q9BEG4	
St6GalNAc-VI	*Bos taurus*	n.d.	AJ620949	CAF06586.1		
CDS4	*Branchiostoma floridae*	n.d.	AF391289	AAM18873.1	Q8T771	
polysialyltransferase (PST) (fragment) ST8Sia IV	*Cercopithecus aethiops*	2.4.99.-	AF210729	AAF17105.1	Q9TT09	
polysialyltransferase (STX) (fragment) ST8Sia II	*Cercopithecus aethiops*	2.4.99.-	AF210318	AAF17104.1	Q9TT10	
α-2,3-sialyltransferase ST3Gal I (Siat4)	*Ciona intestinalis*	n.d.	AJ626815	CAF25173.1		
α-2,3-sialyltransferase ST3Gal I (Siat4)	*Ciona savignyi*	n.d.	AJ626814	CAF25172.1		
α-2,8-polysialyltransferase ST8Sia IV	*Cricetulus griseus*	2.4.99.-	- Z46801	AAE28634 CAA86822.1	Q64690	
Gal β-1,3/4-GlcNAc α-2,3-sialyltransferase St3Gal I	*Cricetulus griseus*	n.d.	AY266675	AAP22942.1	Q80WL0	
Gal β-1,3/4-GlcNAc α-2,3-sialyltransferase St3Gal II (fragment)	*Cricetulus griseus*	n.d.	AY266676	AAP22943.1	Q80WK9	
α-2,3-sialyltransferase ST3Gal I (Siat4)	*Danio rerio*	n.d.	AJ783740	CAH04017.1		
α-2,3-sialyltransferase ST3Gal II (Siat5)	*Danio rerio*	n.d.	AJ783741	CAH04018.1		

FIG. 2B

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / 3D
α-2,3-sialyltransferase ST3Gal III (Siat6)	*Danio rerio*	n.d.	AJ626821	CAF25179.1		
α-2,3-sialyltransferase ST3Gal IV (Siat4c)	*Danio rerio*	n.d.	AJ744809	CAG32845.1		
α-2,3-sialyltransferase ST3Gal V-r (Siat6-related)	*Danio rerio*	n.d.	AJ783742	CAH04019.1		
α-2,6-sialyltransferase ST6Gal I (Siat1)	*Danio rerio*	n.d.	AJ744801	CAG32837.1		
α-2,6-sialyltransferase ST6GalNAc II (Siat7B)	*Danio rerio*	n.d.	AJ634459	CAG25680.1		
α-2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)	*Danio rerio*	n.d.	AJ646874	CAG26703.1		
α-2,6-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)	*Danio rerio*	n.d.	AJ646883	CAG26712.1		
α-2,8-sialyltransferase ST8Sia I (Siat 8A) (fragment)	*Danio rerio*	n.d.	AJ715535	CAG29374.1		
α-2,8-sialyltransferase ST8Sia III (Siat 8C) (fragment)	*Danio rerio*	n.d.	AJ715543	CAG29382.1		
α-2,8-sialyltransferase ST8Sia IV (Siat 8D) (fragment)	*Danio rerio*	n.d.	AJ715545	CAG29384.1		
α-2,8-sialyltransferase ST8Sia V (Siat 8E) (fragment)	*Danio rerio*	n.d.	AJ715546	CAG29385.1		
α-2,8-sialyltransferase ST8Sia VI (Siat 8F) (fragment)	*Danio rerio*	n.d.	AJ715551	CAG29390.1		
β-galactosamide α-2,6-sialyltransferase II (ST6Gal II)	*Danio rerio*	n.d.	AJ627627	CAF29495.1		
N-glycan α-2,8-sialyltransferase	*Danio rerio*	n.d.	BC050483 AY055462 NM_153662	AAH50483.1 AAL17875.1 NP_705948.1	Q7ZU51 Q8QH83	
ST3Gal III-related (siat6r)	*Danio rerio*	n.d.	BC053179 AJ626820 NM_200355	AAH53179.1 CAF25178.1 NP_956649.1	Q7T3B9	
St3Gal-V	*Danio rerio*	n.d.	AJ819960	CAF04061.1		
st6GalNAc-VI	*Danio rerio*	n.d.	BC050932 AJ620947	AAH50932.1 CAF06584.1		
α-2,6-sialyltransferase (CG4871) ST6Gal I	*Drosophila melanogaster*	2.4.99.1	AE003485 AF218237 AF397532 AE003465 NM_079129 NM_166684	AAF47256.1 AAG13165.1 AAK92126.1 AAM70791.1 NP_523853.1 NP_726474.1	Q9GU23 Q9W121	
α-2,3-sialyltransferase (ST3Gal-VI)	*Gallus gallus*	n.d.	AJ585767 AJ627204	CAE51391.1 CAF25503.1		
α-2,3-sialyltransferase ST3Gal I	*Gallus gallus*	2.4.99.4	X80503 NM_205217	CAA56666.1 NP_990548.1	Q11200	
α-2,3-sialyltransferase ST3Gal IV (fragment)	*Gallus gallus*	2.4.99.-	AF035250	AAC14163.1	O73724	
α-2,3-sialyltransferase (ST3GAL-II)	*Gallus gallus*	n.d.	AJ585761	CAE51385.2		
α-2,6-sialyltransferase (Siat7b)	*Gallus gallus*	n.d.	AJ620653	CAF05852.1		
α-2,6-sialyltransferase ST6Gal I	*Gallus gallus*	2.4.99.1	X76558 NM_205241	CAA53235.1 NP_990572.1	Q92182	
α-2,6-sialyltransferase	*Gallus gallus*	2.4.99.3	-	AAE68028.1	Q92183	

FIG. 2C

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
ST6GalNAc I				X74946 NM_205240	AAE68029.1 CAA52902.1 NP_990571.1		
α-2,6-sialyltransferase ST6GalNAc II		*Gallus gallus*	2.4.99.-	X77775 NM_205233	AAE68030.1 CAA54613.1 NP_990564.1	Q92184	
α-2,6-sialyltransferase ST6GalNAc III (SIAT7C) (fragment)		*Gallus gallus*	n.d.	AJ634455	CAG25677.1		
α-2,6-sialyltransferase ST6GalNAc V (SIAT7E) (fragment)		*Gallus gallus*	n.d.	AJ646877	CAG26706.1		
α-2,8-sialyltransferase (GD3 Synthase) ST8Sia I		*Gallus gallus*	2.4.99.-	U73176	AAC28888.1	P79783	
α-2,8-sialyltransferase (SIAT8B)		*Gallus gallus*	n.d.	AJ699419	CAG27881.1		
α-2,8-sialyltransferase (SIAT8C)		*Gallus gallus*	n.d.	AJ699420	CAG27882.1		
α-2,8-sialyltransferase (SIAT8F)		*Gallus gallus*	n.d.	AJ699424	CAG27886.1		
α-2,8-syalyltransferase ST8Siα-V (SIAT8C)		*Gallus gallus*	n.d.	AJ704564	CAG28697.1		
β-galactosamide α-2,6-sialyltransferase II (ST6Gal II)		*Gallus gallus*	n.d.	AJ627629	CAF29497.1		
GM3 synthase (SIAT9)		*Gallus gallus*	2.4.99.9	AY515255	AAS83519.1		
polysialyltransferase ST8Sia IV		*Gallus gallus*	2.4.99.-	AF008194	AAB95120.1	O42399	
α-2,3-sialyltransferase ST3Gal I		*Homo sapiens*	2.4.99.4	L29555 AF059321 L13972 AF155238 AF186191 BC018357 NM_003033 NM_173344	AAA36612.1 AAC17874.1 AAC37574.1 AAD39238.1 AAG29876.1 AAH18357.1 NP_003024.1 NP_775479.1	Q11201 O60877 Q9UN51	
α-2,3-sialyltransferase ST3Gal II		*Homo sapiens*	2.4.99.4	U63090 BC036777 X96667 NM_006927	AAB40389.1 AAH36777.1 CAA65447.1 NP_008858.1	Q16842 O00654	
α-2,3-sialyltransferase ST3Gal III (SIaT6)		*Homo sapiens*	2.4.99.6	L23768 BC050380 AF425851 AF425852 AF425853 AF425854 AF425855 AF425856 AF425857 AF425858 AF425859 AF425860 AF425861 AF425862 AF425863 AF425864 AF425865 AF425866 AF425867 AY167992 AY167993 AY167994	AAA35778.1 AAH50380.1 AAO13859.1 AAO13860.1 AAO13861.1 AAO13862.1 AAO13863.1 AAO13864.1 AAO13865.1 AAO13866.1 AAO13867.1 AAO13868.1 AAO13869.1 AAO13870.1 AAO13871.1 AAO13872.1 AAO13873.1 AAO13874.1 AAO13875.1 AAO38806.1 AAO38807.1 AAO38808.1	Q11203 Q86UR6 Q86UR7 Q86UR8 Q86UR9 Q86US0 Q86US1 Q86US2 Q8IX43 Q8IX44 Q8IX45 Q8IX46 Q8IX47 Q8IX48 Q8IX49 Q8IX50 Q8IX51 Q8IX52 Q8IX53 Q8IX54 Q8IX55 Q8IX56	

FIG. 2D

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
				AY167995	AAO38809.1	Q8IX57	
				AY167996	AAO38810.1	Q8IX58	
				AY167997	AAO38811.1		
				AY167998	AAO38812.1		
				NM_006279	NP_006270.1		
				NM_174964	NP_777624.1		
				NM_174965	NP_777625.1		
				NM_174966	NP_777626.1		
				NM_174967	NP_777627.1		
				NM_174969	NP_777629.1		
				NM_174970	NP_777630.1		
				NM_174972	NP_777632.1		
α-2,3-sialyltransferase ST3Gal IV		*Homo sapiens*	2.4.99.-	L23767	AAA16460.1	Q11206	
				AF035249	AAC14162.1	O60497	
				BC010645	AAH10645.1	Q96QQ9	
				AY040826	AAK93790.1	Q8N6A6	
				AF516602	AAM66431.1	Q8N6A7	
				AF516603	AAM66432.1	Q8NFD3	
				AF516604	AAM66433.1	Q8NFG7	
				AF525084	AAM81378.1		
				X74570	CAA52662.1		
				CR456858	CAG33139.1		
				NM_006278	NP_006269.1		
α-2,3-sialyltransferase ST3Gal VI		*Homo sapiens*	2.4.99.4	AF119391	AAD39131.1	Q9Y274	
				BC023312	AAH23312.1		
				AB022918	BAA77609.1		
				AX877628	CAE89895.1		
				AX886023	CAF00161.1		
				NM_006100	NP_006091.1		
α-2,6-sialyltransferase (ST6Gal II ; KIAA1877)		*Homo sapiens*	n.d.	BC008680	AAH08680.1	Q86Y44	
				AB058780	BAB47506.1	Q8IUG7	
				AB059555	BAC24793.1	Q96HE4	
				AJ512141	CAD54408.1	Q96JF0	
				AX795193	CAE48260.1		
				AX795193	CAE48261.1		
				NM_032528	NP_115917.1		
α-2,6-sialyltransferase (ST6GALNAC III)		*Homo sapiens*	n.d.	BC059363	AAH59363.1	Q8N259	
				AY358540	AAQ88904.1	Q8NDV1	
				AK091215	BAC03611.1		
				AJ507291	CAD45371.1		
				NM_152996	NP_694541.1		
α-2,6-sialyltransferase (ST6GalNAc V)		*Homo sapiens*	n.d.	BC001201	AAH01201.1	Q9BVH7	
				AK056241	BAB71127.1		
				AL035409	CAB72344.1		
				AJ507292	CAD45372.1		
				NM_030965	NP_112227.1		
α-2,6-sialyltransferase (SThM) ST6GalNAc II		*Homo sapiens*	2.4.99.-	U14550	AAA52228.1	Q9UJ37	
				BC040455	AAH40455.1	Q12971	
				AJ251053	CAB61434.1		
				NM_006456	NP_006447.1		
α-2,6-sialyltransferase ST6Gal I		*Homo sapiens*	2.4.99.1	BC031476	AAH31476.1	P15907	
				BC040009	AAH40009.1		
				A17362	CAA01327.1		
				A23699	CAA01686.1		
				X17247	CAA35111.1		
				X54363	CAA38246.1		
				X62822	CAA44634.1		
				NM_003032	NP_003023.1		
				NM_173216	NP_775323.1		
α-2,6-sialyltransferase ST6GalNAc I		*Homo sapiens*	2.4.99.3	BC022462	AAH22462.1	Q8TBJ6	
				AY096001	AAM22800.1	Q9NSC7	
				AY358918	AAQ89277.1	Q9NXQ7	
				AK000113	BAA90953.1		
				Y11339	CAA72179.2		

FIG. 2E

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / 3D
			NM_018414	NP_060884.1		
α-2,8-polysialyltransferase ST8Sia IV	*Homo sapiens*	2.4.99.-	L41680 BC027886 BC053857 NM_005668	AAC41775.1 AAH27886.1 AAH53657.1 NP_005659.1	Q8N1F4 Q92187 Q92693	
α-2,8-sialyltransferase (GD3 synthase) ST8Sia I	*Homo sapiens*	2.4.99.8	L32867 L43494 BC046158 - AY569975 D26360 X77922 NM_003034	AAA62366.1 AAC37586.1 AAH46158.1 AAQ53140.1 AAS75783.1 BAA05391.1 CAA54891.1 NP_003025.1	Q86X71 Q92185 Q93064	
α-2,8-sialyltransferase ST8Sia II	*Homo sapiens*	2.4.99.-	L29556 U82762 U33551 BC069584 NM_005011	AAA36613.1 AAB51242.1 AAC24456.1 AAH69584.1 NP_005002.1	Q92186 Q92470 Q92746	
α-2,8-sialyltransferase ST8Sia III	*Homo sapiens*	2.4.99.-	AF004668 AF003092 NM_015879	AAB87642.1 AAC15901.2 NP_056983.1	O43173 Q9NS41	
α-2,8-sialyltransferase ST8Sia V	*Homo sapiens*	2.4.99.-	U91641 CR457037 NM_013305	AAC51727.1 CAG33318.1 NP_037437.1	O15466	
ENSP00000020221 (fragment)		n.d.	AC023295	-		
lactosylceramide α-2,3-sialyltransferase (ST3Gal V)	*Homo sapiens*	2.4.99.9	AF105026 AF119415 BC065936 AY152815 AAP65066 AY359105 AB018358 AX876536 NM_003896	AAD14634.1 AAF66146.1 AAH65936.1 AAO16866.1 AAP65066.1 AAQ89463.1 BAA33950.1 CAE89320.1 NP_003887.2	Q9UNP4 O94902	
N-acetylgalactosaminide α-2,6-sialyltransferase (ST6GalNAc VI)	*Homo sapiens*	2.4.99.-	BC006564 BC007802 BC016299 AY358672 AB035173 AK023900 AJ507293 AX880950 CR457318 NM_013443	AAH06564.1 AAH07802.1 AAH16299.1 AAQ89035.1 BAA87035.1 BAB14715.1 CAD45373.1 CAE91145.1 CAG33599.1 NP_038471.2	Q969X2 Q9H8A2 Q9ULB8	
N-acetylgalactosaminide α-2,6-sialyltransferase IV (ST6GalNAc IV)	*Homo sapiens*	2.4.99.-	AF127142 BC036705 - AB035172 AK000600 Y17461 AJ271734 AX061620 AX068265 AX969252 NM_014403 NM_175039	AAF00102.1 AAH36705.1 AAP63349.1 BAA87034.1 BAA91281.1 CAB44354.1 CAC07404.1 CAC24981.1 CAC27250.1 CAF14360.1 NP_055216.3 NP_778204.1	Q9H4F1 Q9NWU6 Q9UKU1 Q9ULB9 Q9Y3G3 Q9Y3G4	
ST8SIA-VI (fragment)	*Homo sapiens*	n.d.	AJ621583 XM_291725	CAF21722.1 XP_291725.2		
unnamed protein product	*Homo sapiens*	n.d.	AK021929 AX881696	BAB13940.1 CAE91353.1	Q9HAA9	
Gal β-1,3/4-GlcNAc α-	*Mesocricetus*	2.4.99.6	AJ245699	CAB53394.1	Q9QXF6	

FIG. 2F

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
2,3-sialyltransferase (ST3Gal III)		*auratus*					
Gal β-1,3/4-GlcNAc α-2,3-sialyltransferase (ST3Gal IV)		*Mesocricetus auratus*	2.4.99.6	AJ245700	CAB53395.1	Q9QXF5	
GD3 synthase (fragment) ST8Sia I		*Mesocricetus auratus*	n.d.	AF141657	AAD33979.1	Q9WUL1	
polysialyltransferase (ST8Sia IV)		*Mesocricetus auratus*	2.4.99.-	AJ245701	CAB53396.1	Q9QXF4	
α-2,3-sialyltransferase ST3Gal I	*St3gal1*	*Mus musculus*	2.4.99.4	AF214028 AK031344 AK078469 X73523 NM_009177	AAF60973.1 BAC27356.1 BAC37290.1 CAA51919.1 NP_033203.1	P54751 Q11202 Q9JL30	
α-2,3-sialyltransferase ST3Gal II	*St3gal2*	*Mus musculus*	2.4.99.4	BC015264 BC066064 AK034554 AK034863 AK053827 X76989 NM_009179 NM_178048	AAH15264.1 AAH66064.1 BAC28752.1 BAC28859.1 BAC35543.1 CAA54294.1 NP_033205.1 NP_835149.1	Q11204 Q8BPL0 Q8BSA0 Q8BSE9 Q91WH6	
α-2,3-sialyltransferase ST3Gal III	*St3gal3*	*Mus musculus*	2.4.99.-	BC006710 AK005053 AK013016 X84234 NM_009176	AAH06710.1 BAB23779.1 BAB28598.1 CAA59013.1 NP_033202.2	P97325 Q922X5 Q9CZ48 Q9DBB6	
α-2,3-sialyltransferase ST3Gal IV	*St3gal4*	*Mus musculus*	2.4.99.4	BC011121 BC050773 D28941 AK008543 AB061305 X95809 NM_009178	AAH11121.1 AAH50773.1 BAA06068.1 BAB25732.1 BAB47508.1 CAA65076.1 NP_033204.2	P97354 Q61325 Q91Y74 Q921R5 Q9CVE8	
α-2,3-sialyltransferase ST3Gal VI	*St3gal6*	*Mus musculus*	2.4.99.4	AF119390 BC052338 AB063326 AK033562 AK041173 NM_018784	AAD39130.1 AAH52338.1 BAB79494.1 BAC28360.1 BAC30851.1 NP_061254	Q80UR7 Q8BLV1 Q8VIB3 Q9WVG2	
α-2,6-sialyltransferase ST6GalNAc II	*St6galnac2*	*Mus musculus*	2.4.99.-	NM_009180 BC010208 AB027198 AK004613 X93999 X94000 NM_009180	6677963 AAH10208.1 BAB00637.1 BAB23410.1 CAA63821.1 CAA63822.1 NP_033206.2	P70277 Q9DC24 Q9JJM5	
α-2,6-sialyltransferase ST6Gal I	*St6gal1*	*Mus musculus*	2.4.99.1	- BC027833 D16106 AK034768 AK084124 NM_145933	AAE68031.1 AAH27833.1 BAA03680.1 BAC28828.1 BAC39120.1 NP_666045.1	Q64685 Q8BM62 Q8K1L1	
α-2,6-sialyltransferase ST6Gal II	*St6gal2*	*Mus musculus*	n.d.	AK082566 AB095093 AK129462 NM_172829	BAC38534.1 BAC87752.1 BAC98272.1 NP_766417.1	Q8BUU4	
α-2,6-sialyltransferase ST6GalNAc I	*St6galnac1*	*Mus musculus*	2.4.99.3	Y11274 NM_011371	CAA72137.1 NP_035501.1	Q9QZ39 Q8JJP5	
α-2,6-sialyltransferase ST6GalNAc III	*St6galnac3*	*Mus musculus*	n.d.	BC058387 AK034804 Y11342 Y11343	AAH58387.1 BAC28836.1 CAA72181.2 CAB95031.1	Q9WUV2 Q9JHP5	

FIG. 2G

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
				NM_011372	NP_035502		
α-2,6-sialyltransferase ST6GalNAc IV	*St6galnac4*	*Mus musculus*	2.4.99.7	BC056451	AAH56451.1	Q8C3J2	
				AK085730	BAC39523.1	Q8JHP2	
				AJ007310	CAA07446.1	Q9R2B6	
				Y15779	CAB43507.1	O88725	
				Y15780	CAB43514.1	Q9JHP0	
				Y19055	CAB93946.1	Q9QUP9	
				Y19057	CAB93948.1	Q9R2B5	
				NM_011373	NP_035503.1		
α-2,8-sialyltransferase (GD3 synthase) ST8Sia I	*St8sia1*	*Mus musculus*	2.4.99.8	L38677	AAA91869.1	Q64468	
				BC024821	AAH24821.1	Q64687	
				AK046188	BAC32625.1	Q8BL76	
				AK052444	BAC34994.1	Q8BWI0	
				X84235	CAA59014.1	Q8K1C1	
				AJ401102	CAC20706.1	Q9EPK0	
				NM_011374	NP_035504.1		
α-2,8-sialyltransferase (ST8Sia VI)	*St8sia6*	*Mus musculus*	n.d.	AB059554	BAC01265.1	Q8BI43	
				AK085105	BAC39367.1	Q8K4T1	
				NM_145838	NP_665837.1		
α-2,8-sialyltransferase ST8Sia II	*St8sia2*	*Mus musculus*	2.4.99.-	X83562	CAA58548.1	O35696	
				X99646	CAA67965.1		
				X99647	CAA67965.1		
				X99648	CAA67965.1		
				X99649	CAA67965.1		
				X99650	CAA67965.1		
				X99651	CAA67965.1		
				NM_009181	NP_033207.1		
α-2,8-sialyltransferase ST8Sia IV	*St8sia4*	*Mus musculus*	2.4.99.8	BC060112	AAH60112.1	Q64692	
				AK003690	BAB22941.1	Q8BY70	
				AK041723	BAC31044.1		
				AJ223956	CAA11685.1		
				X85000	CAA59992.1		
				Y09484	CAA70692.1		
				NM_009183	NP_033209.1		
α-2,8-sialyltransferase ST8Sia V	*St8sia5*	*Mus musculus*	2.4.99.-	BC034855	AAH34855.1	P70126	
				AK078670	BAC37354.1	P70127	
				X98014	CAA66642.1	P70128	
				X98014	CAA66643.1	Q8BJW0	
				X98014	CAA66644.1	Q8JZQ3	
				NM_013666	NP_038694.1		
				NM_153124	NP_694764.1		
				NM_177416	NP_803135.1		
α-2,8-sialyltransferase ST8Sia III	*St8sia3*	*Mus musculus*	2.4.99.-	BC075645	AAH75645.1	Q64689	
				AK015874	BAB30012.1	Q9CUJ6	
				X80502	CAA56665.1		
				NM_009182	NP_033208.1		
GD1 synthase (ST6GalNAc V)	*St6galnac5*	*Mus musculus*	n.d.	BC055737	AAH55737.1	Q8CAM7	
				AB030836	BAA85747.1	Q8CBX1	
				AB028840	BAA89292.1	Q9QYJ1	
				AK034387	BAC28693.1	Q9R0K6	
				AK038434	BAC29997.1		
				AK042683	BAC31331.1		
				NM_012028	NP_036158.2		
GM3 synthase (α-2,3-sialyltransferase) ST3Gal V	*St3gal5*	*Mus musculus*	2.4.99.9	AF119416	AAF66147.1	O88829	
				-	AAP65083.1	Q9CZ65	
				AB018048	BAA33491.1	Q9QWF9	
				AB013302	BAA76467.1		
				AK012961	BAB28571.1		
				Y15003	CAA75235.1		
				NM_011375	NP_035505.1		
N-acetylgalactosaminide α-2,6-sialyltransferase (ST6GalNAc VI)	*St6galnac6*	*Mus musculus*	2.4.99.-	BC036985	AAH36985.1	Q8CDC3	
				AB035174	BAA87036.1	Q8JZW3	
				AB035123	BAA95940.1	Q9JM95	
				AK030648	BAC27064.1	Q9R0G9	

FIG. 2H

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
				NM_016973	NP_058669.1		
M138L		*Myxoma virus*	n.d.	U46578 AF170726 NC_001132	AAD00069.1 AAE61323.1 AAE61326.1 AAF15026.1 NP_051852.1		
α-2,3-sialyltransferase (St3Gal-I)		*Oncorhynchus mykiss*	n.d.	AJ585760	CAE51384.1		
α-2,6-sialyltransferase (Siat1)		*Oncorhynchus mykiss*	n.d.	AJ620649	CAF05848.1		
α-2,8-polysialyltransferase IV (ST8Sia IV)		*Oncorhynchus mykiss*	n.d.	AB094402	BAC77411.1	Q7T2X5	
GalNAc α-2,6-sialyltransferase (RtST6GalNAc)		*Oncorhynchus mykiss*	n.d.	AB097943	BAC77520.1	Q7T2X4	
α-2,3-sialyltransferase ST3Gal IV		*Oryctolagus cuniculus*	2.4.99.-	AF121967	AAF26871.1	Q9N257	
OJ1217_F02.7		*Oryza sativa (japonica cultivar-group)*	n.d.	AP004084	BAD07616.1		
OSJNBa0043L24.2 or OSJNBb0002J11.9		*Oryza sativa (japonica cultivar-group)*	n.d.	AL731626 AL662969	CAD41185.1 CAE04714.1		
P0683f02.18 or P0489B03.1		*Oryza sativa (japonica cultivar-group)*	n.d.	AP003289 AP003794	BAB63715.1 BAB90552.1		
α-2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)		*Oryzias latipes*	n.d.	AJ646876	CAG26705.1		
α-2,3-sialyltransferase ST3Gal I (Siat4)		*Pan troglodytes*	n.d.	AJ744803	CAG32839.1		
α-2,3-sialyltransferase ST3Gal II (Siat5)		*Pan troglodytes*	n.d.	AJ744804	CAG32840.1		
α-2,3-sialyltransferase ST3Gal III (Siat6)		*Pan troglodytes*	n.d.	AJ626819	CAF25177.1		
α-2,3-sialyltransferase ST3Gal IV (Siat4c)		*Pan troglodytes*	n.d.	AJ626824	CAF25182.1		
α-2,3-sialyltransferase ST3Gal VI (Siat10)		*Pan troglodytes*	n.d.	AJ744808	CAG32844.1		
α-2,6-sialyltransferase (Sia7A)		*Pan troglodytes*	n.d.	AJ748740	CAG33615.1		
α-2,6-sialyltransferase (Sia7B)		*Pan troglodytes*	n.d.	AJ748741	CAG33616.1		
α-2,6-sialyltransferase ST6GalNAc III (Siat7C)		*Pan troglodytes*	n.d.	AJ634454	CAG25676.1		
α-2,6-sialyltransferase ST6GalNAc IV (Siat7D) (fragment)		*Pan troglodytes*	n.d.	AJ646870	CAG26699.1		
α-2,6-sialyltransferase ST6GalNAc V (Siat7E)		*Pan troglodytes*	n.d.	AJ646875	CAG26704.1		
α-2,6-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)		*Pan troglodytes*	n.d.	AJ646882	CAG26711.1		
α-2,8-sialyltransferase 8A (Siat8A)		*Pan troglodytes*	2.4.99.8	AJ697658	CAG26896.1		
α-2,8-sialyltransferase 8B (Siat8B)		*Pan troglodytes*	n.d.	AJ697659	CAG26897.1		
α-2,8-sialyltransferase 8C (Siat8C)		*Pan troglodytes*	n.d.	AJ697660	CAG26898.1		
α-2,8-sialyltransferase 8D (Siat8D)		*Pan troglodytes*	n.d.	AJ697661	CAG26899.1		
α-2,8-sialyltransferase		*Pan troglodytes*	n.d.	AJ697662	CAG26900.1		

FIG. 2I

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / 3D
8E (Siat8E)						
α-2,8-sialyltransferase 8F (Siat8F)	*Pan troglodytes*	n.d.	AJ697663	CAQ26901.1		
β-galactosamide α-2,6-sialyltransferase I (ST6Gal I; Siat1)	*Pan troglodytes*	2.4.99.1	AJ627624	CAF29492.1		
β-galactosamide α-2,6-sialyltransferase II (ST6Gal II)	*Pan troglodytes*	n.d.	AJ627625	CAF29493.1		
GM3 synthase ST3Gal V (Siat9)	*Pan troglodytes*	n.d.	AJ744807	CAG32843.1		
S138L	*Rabbit fibroma virus Kasza*	n.d.	NC_001266	NP_052025		
α-2,3-sialyltransferase ST3Gal III	*Rattus norvegicus*	2.4.99.6	M97754 NM_031697	AAA42146.1 NP_113885.1	Q02734	
α-2,3-sialyltransferase ST3Gal IV (Siat4c)	*Rattus norvegicus*	n.d.	AJ626825	CAF25183.1		
α-2,3-sialyltransferase ST3Gal VI	*Rattus norvegicus*	n.d.	AJ626743	CAF25053.1		
α-2,6-sialyltransferase ST3Gal II	*Rattus norvegicus*	2.4.99.-	X76988 NM_031695	CAA54293.1 NP_113883.1	Q11205	
α-2,6-sialyltransferase ST6Gal I	*Rattus norvegicus*	2.4.99.1	M18769 M83143	AAA41196.1 AAB07233.1	P13721	
α-2,6-sialyltransferase ST6GalNAc I (Siat7A)	*Rattus norvegicus*	n.d.	AJ634458	CAG25684.1		
α-2,6-sialyltransferase ST6GalNAc II (Siat7B)	*Rattus norvegicus*	n.d.	AJ634457	CAG25679.1		
α-2,6-sialyltransferase ST6GalNAc III	*Rattus norvegicus*	2.4.99.-	L29554 BC072501 NM_019123	AAC42086.1 AAH72501.1 NP_061996.1	Q64686	
α-2,6-sialyltransferase ST6GalNAc IV (Siat7D) (fragment)	*Rattus norvegicus*	n.d.	AJ646871	CAG26700.1		
α-2,6-sialyltransferase ST6GalNAc V (Siat7E)	*Rattus norvegicus*	n.d.	AJ646872	CAG26701.1		
α-2,6-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)	*Rattus norvegicus*	n.d.	AJ646881	CAG26710.1		
α-2,8-sialyltransferase (GD3 synthase) ST8Sia I	*Rattus norvegicus*	2.4.99.-	U53883 D45255	AAC27541.1 BAA08213.1	P70554 P97713	
α-2,8-sialyltransferase (SIAT8E)	*Rattus norvegicus*	n.d.	AJ699422	CAG27684.1		
α-2,8-sialyltransferase (SIAT8F)	*Rattus norvegicus*	n.d.	AJ699423	CAG27685.1		
α-2,8-sialyltransferase ST8Sia II	*Rattus norvegicus*	2.4.99.-	L13445 NM_057156	AAA42147.1 NP_476497.1	Q07977 Q64688	
α-2,8-sialyltransferase ST8Sia III	*Rattus norvegicus*	2.4.99.-	U55938 NM_013029	AAB50061.1 NP_037161.1	P97877	
α-2,8-sialyltransferase ST8Sia IV	*Rattus norvegicus*	2.4.99.-	U90215	AAB49989.1	O08563	
β-galactosamide α-2,6-sialyltransferase II (ST6Gal II)	*Rattus norvegicus*	n.d.	AJ627626	CAF29494.1		
GM3 synthase ST3Gal V	*Rattus norvegicus*	n.d.	AB018049 NM_031337	BAA33492.1 NP_112627.1	O88830	

FIG. 2J

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / 3D
sialyltransferase ST3Gal-I (Siat4A)	*Rattus norvegicus*	n.d.	AJ748840	CAG44449.1		
α-2,3-sialyltransferase (St3Gal-II)	*Silurana tropicalis*	n.d.	AJ585763	CAE51387.1		
α-2,6-sialyltransferase (Siat7b)	*Silurana tropicalis*	n.d.	AJ620650	CAF05849.1		
α-2,6-sialyltransferase (St6galnac)	*Strongylocentrotus purpuratus*	n.d.	AJ699425	CAG27887.1		
α-2,3-sialyltransferase (ST3GAL-III)	*Sus scrofa*	n.d.	AJ585765	CAE51389.1		
α-2,3-sialyltransferase (ST3GAL-IV)	*Sus scrofa*	n.d.	AJ584674	CAE48299.1		
α-2,3-sialyltransferase ST3Gal I	*Sus scrofa*	2.4.99.4	M97753	AAA31125.1	Q02745	
α-2,6-sialyltransferase (fragment) ST6Gal I	*Sus scrofa*	2.4.99.1	AF136746	AAD33059.1	Q9XSG8	
N-galactosamide α-2,6-sialyltransferase (ST6GalNAc-V)	*Sus scrofa*	n.d.	AJ620948	CAF06585.2		
sialyltransferase (fragment) ST6Gal I	*sus scrofa*	n.d.	AF041031	AAC15833.1	O62717	
ST6GALNAC-V	*Sus scrofa*	n.d.	AJ620948	CAF06585.1		
α-2,3-sialyltransferase (Siat5-r)	*Takifugu rubripes*	n.d.	AJ744805	CAG32841.1		
α-2,3-sialyltransferase ST3Gal I (Siat4)	*Takifugu rubripes*	n.d.	AJ626816	CAF25174.1		
α-2,3-sialyltransferase ST3Gal II (Siat5) (fragment)	*Takifugu rubripes*	n.d.	AJ626817	CAF25175.1		
α-2,3-sialyltransferase ST3Gal III (Siat6)	*Takifugu rubripes*	n.d.	AJ626818	CAF25176.1		
α-2,6-sialyltransferase ST6Gal I (Siat1)	*Takifugu rubripes*	n.d.	AJ744800	CAG32836.1		
α-2,6-sialyltransferase ST6GalNAc II (Siat7B)	*Takifugu rubripes*	n.d.	AJ634460	CAG25681.1		
α-2,6-sialyltransferase ST6GalNAc II B (Siat7B-related)	*Takifugu rubripes*	n.d.	AJ634461	CAG25682.1		
α-2,6-sialyltransferase ST6GalNAc III (Siat7C) (fragment)	*Takifugu rubripes*	n.d.	AJ634456	CAG25678.1		
α-2,6-sialyltransferase ST6GalNAc IV (siat7D) (fragment)	*Takifugu rubripes*	2.4.99.3	Y17466 AJ646869	CAB44338.1 CAG26698.1	Q9W6U6	
α-2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)	*Takifugu rubripes*	n.d.	AJ646873	CAG26702.1		
α-2,6-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)	*Takifugu rubripes*	n.d.	AJ646880	CAG26709.1		
α-2,8-sialyltransferase ST8Sia I (Siat 8A) (fragment)	*Takifugu rubripes*	n.d.	AJ715534	CAG29373.1		
α-2,8-sialyltransferase ST8Sia II (Siat 8B) (fragment)	*Takifugu rubripes*	n.d.	AJ715538	CAG29377.1		
α-2,8-sialyltransferase ST8Sia III (Siat 8C) (fragment)	*Takifugu rubripes*	n.d.	AJ715541	CAG29380.1		
α-2,8-sialyltransferase ST8Sia IIIr (Siat 8Cr)	*Takifugu rubripes*	n.d.	AJ715542	CAG29381.1		
α-2,8-sialyltransferase ST8Sia V (Siat 8E)	*Takifugu rubripes*	n.d.	AJ715547	CAG29386.1		

FIG. 2K

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / 3D
(fragment)						
α-2,8-sialyltransferase ST8Sia VI (Siat 8F) (fragment)	*Takifugu rubripes*	n.d.	AJ715549	CAG29388.1		
α-2,8-sialyltransferase ST8Sia VIr (Siat 8Fr)	*Takifugu rubripes*	n.d.	AJ715550	CAG29389.1		
α-2,3-sialyltransferase (Siat5-r)	*Tetraodon nigroviridis*	n.d.	AJ744806	CAG32842.1		
α-2,3-sialyltransferase ST3Gal I (Siat4)	*Tetraodon nigroviridis*	n.d.	AJ744802	CAG32838.1		
α-2,3-sialyltransferase ST3Gal III (Siat6)	*Tetraodon nigroviridis*	n.d.	AJ626822	CAF25180.1		
α-2,6-sialyltransferase ST6GalNAc II (Siat7B)	*Tetraodon nigroviridis*	n.d.	AJ634462	CAG25683.1		
α-2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)	*Tetraodon nigroviridis*	n.d.	AJ646879	CAG26708.1		
α-2,8-sialyltransferase ST8Sia I (Siat 8A) (fragment)	*Tetraodon nigroviridis*	n.d.	AJ715536	CAG29375.1		
α-2,8-sialyltransferase ST8Sia II (Siat 8B) (fragment)	*Tetraodon nigroviridis*	n.d.	AJ715537	CAG29376.1		
α-2,8-sialyltransferase ST8Sia III (Siat 8C) (fragment)	*Tetraodon nigroviridis*	n.d.	AJ715539	CAG29378.1		
α-2,8-sialyltransferase ST8Sia IIIr (Siat 8Cr) (fragment)	*Tetraodon nigroviridis*	n.d.	AJ715540	CAG29379.1		
α-2,8-sialyltransferase ST8Sia V (Siat 8E) (fragment)	*Tetraodon nigroviridis*	n.d.	AJ715548	CAG29387.1		
α-2,3-sialyltransferase (St3Gal-II)	*Xenopus laevis*	n.d.	AJ585762	CAE51386.1		
α-2,3-sialyltransferase (St3Gal-VI)	*Xenopus laevis*	n.d.	AJ585766	CAE51390.1		
α-2,3-sialyltransferase St3Gal-III (Siat6)	*Xenopus laevis*	n.d.	AJ585764 AJ626823	CAE51388.1 CAF25181.1		
α-2,8-polysialyltransferase	*Xenopus laevis*	2.4.99.-	AB007468	BAA32617.1	O93234	
α-2,8-sialyltransferase ST8Siα-I (Siat8A;GD3 synthase)	*Xenopus laevis*	n.d.	AY272056 AY272057 AJ704562	AAQ16162.1 AAQ16163.1 CAG28695.1		
Unknown (protein for MGC:81265)	*Xenopus laevis*	n.d.	BC068760	AAH68760.1		
α-2,3-sialyltransferase (3Gal-VI)	*Xenopus tropicalis*	n.d.	AJ626744	CAF25054.1		
α-2,3-sialyltransferase (Siat4c)	*Xenopus tropicalis*	n.d.	AJ622908	CAF22058.1		
α-2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)	*Xenopus tropicalis*	n.d.	AJ646878	CAG26707.1		
α-2,8-sialyltransferase ST8Sia III (Siat 8C) (fragment)	*Xenopus tropicalis*	n.d.	AJ715544	CAG29383.1		
β-galactosamide α-2,6-sialyltransferase II (ST6Gal II)	*Xenopus tropicalis*	n.d.	AJ627628	CAF29496.1		
sialytransferase St8Sia1	*Xenopus tropicalis*	n.d.	AY652775	AAT67042		
poly-α-2,8-sialosyl sialyltransferase (NeuS)	*Escherichia coli K1*	2.4.-.-	M76370 X60598	AAA24213.1 CAA43053.1	Q57269	
polysialyltransferase	*Escherichia coli K92*	2.4.-.-	M88479	AAA24215.1	Q47404	

FIG. 2L

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB /3D
α-2,8 polysialyltransferase SiaD	*Neisseria meningitidis B1940*	2.4.-.-	M95053 X78068	AAA20478.1 CAA54985.1	Q51281 Q51145	
SynE	*Neisseria meningitidis FAM18*	n.d.	U75650	AAB53842.1	O06435	
polysialyltransferase (SiaD)(fragment)	*Neisseria meningitidis M1019*	n.d.	AY234192	AAO85290.1		
SiaD (fragment)	*Neisseria meningitidis M209*	n.d.	AY281046	AAP34768.1		
SiaD (fragment)	*Neisseria meningitidis M3045*	n.d.	AY281044	AAP34767.1		
polysialyltransferase (SiaD)(fragment)	*Neisseria meningitidis M3315*	n.d.	AY234191	AAO85289.1		
SiaD (fragment)	*Neisseria meningitidis M3515*	n.d.	AY281047	AAP34770.1		
polysialyltransferase (SiaD)(fragment)	*Neisseria meningitidis M4211*	n.d.	AY234190	AAO85288.1		
SiaD (fragment)	*Neisseria meningitidis M4642*	n.d.	AY281048	AAP34771.1		
polysialyltransferase (SiaD)(fragment)	*Neisseria meningitidis M5177*	n.d.	AY234193	AAO85291.1		
SiaD	*Neisseria meningitidis M5178*	n.d.	AY281049	AAP34766.1		
SiaD (fragment)	*Neisseria meningitidis M980*	n.d.	AY281045	AAP34769.1		
NMB0067	*Neisseria meningitidis MC58*	n.d.	NC_003112	NP_273131		
Lst	*Aeromonas punctata Sch3*	n.d.	AF126256	AAS66624.1		
ORF2	*Haemophilus influenzae A2*	n.d.	M94855	AAA24979.1		
HI1699	*Haemophilus influenzae Rd*	n.d.	U32842 NC_000907	AAC23345.1 NP_439841.1	Q48211	
α-2,3-sialyltransferase	*Neisseria gonorrhoeae F62*	2.4.99.4	U60664	AAC44539.1 AAE67205.1	P72074	
α-2,3-sialyltransferase	*Neisseria meningitidis 126E, NRCC 4010*	2.4.99.4	U60662	AAC44544.2		
α-2,3-sialyltransferase	*Neisseria meningitidis 406Y, NRCC 4030*	2.4.99.4	U60661	AAC44543.1		
α-2,3-sialyltransferase (NMB0922)	*Neisseria meningitidis MC58*	2.4.99.4	U60660 AE002443 NC_003112	AAC44541.1 AAF41330.1 NP_273962.1	P72097	
NMA1118	*Neisseria meningitidis Z2491*	n.d.	AL162755 NC_003116	CAB84380.1 NP_283887.1	Q9JUV5	
PM0508	*Pasteurella multocida PM70*	n.d.	AE006086 NC_002663	AAK02592.1 NP_245445.1	Q9CNC4	
WaaH	*Salmonella enterica SARB25*	n.d.	AF519787	AAM82550.1	Q8KS93	
WaaH	*Salmonella enterica SARB3*	n.d.	AF519788	AAM82551.1	Q8KS92	
WaaH	*Salmonella enterica SARB39*	n.d.	AF519789	AAM82552.1		
WaaH	*Salmonella enterica SARB53*	n.d.	AF519790	AAM82553.1		
WaaH	*Salmonella enterica SARB57*	n.d.	AF519791	AAM82554.1	Q8KS91	
WaaH	*Salmonella enterica SARB71*	n.d.	AF519793	AAM82556.1	Q8KS89	
WaaH	*Salmonella enterica*	n.d.	AF519792	AAM82555.1	Q8KS90	

FIG. 2M

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / 3D
	SARB8					
WaaH	*Salmonella enterica SARC10V*	n.d.	AF519779	AAM88840.1	Q8KS99	
WaaH (fragment)	*Salmonella enterica SARC12*	n.d.	AF519781	AAM88842.1		
WaaH (fragment)	*Salmonella enterica SARC13I*	n.d.	AF519782	AAM88843.1	Q8KS98	
WaaH (fragment)	*Salmonella enterica SARC14I*	n.d.	AF519783	AAM88844.1	Q8KS97	
WaaH	*Salmonella enterica SARC15II*	n.d.	AF519784	AAM88845.1	Q8KS96	
WaaH	*Salmonella enterica SARC16II*	n.d.	AF519785	AAM88846.1	Q8KS95	
WaaH (fragment)	*Salmonella enterica SARC3I*	n.d.	AF519772	AAM88834.1	Q8KSA4	
WaaH (fragment)	*Salmonella enterica SARC4I*	n.d.	AF519773	AAM88835.1	Q8KSA3	
WaaH	*Salmonella enterica SARC5IIa*	n.d.	AF519774	AAM88836.1		
WaaH	*Salmonella enterica SARC6IIa*	n.d.	AF519775	AAM88837.1	Q8KSA2	
WaaH	*Salmonella enterica SARC8*	n.d.	AF519777	AAM88838.1	Q8KSA1	
WaaH	*Salmonella enterica SARC9V*	n.d.	AF519778	AAM88839.1	Q8KSA0	
UDP-glucose : α-1,2-glucosyltransferase (WaaH)	*Salmonella enterica subsp. arizonae SARC 5*	2.4.1.-	AF511116	AAM48166.1		
bifunctional α-2,3/-2,8-sialyltransferase (Cst-II)	*Campylobacter jejuni ATCC 43449*	n.d.	AF401529	AAL06004.1	Q93CZ5	
Cst	*Campylobacter jejuni 81-176*	n.d.	AF305571	AAL09368.1		
α-2,3-sialyltransferase (Cst-III)	*Campylobacter jejuni ATCC 43429*	2.4.99.-	AY044156	AAK73183.1		
α-2,3-sialyltransferase (Cst-III)	*Campylobacter jejuni ATCC 43430*	2.4.99.-	AF400047	AAK85419.1		
α-2,3-sialyltransferase (Cst-II)	*Campylobacter jejuni ATCC 43432*	2.4.99.-	AF215659	AAG43979.1	Q9F0M9	
α-2,3/8-sialyltransferase (CstII)	*Campylobacter jejuni ATCC 43438*	n.d.	AF400048	AAK91725.1	Q93MQ0	
α-2,3-sialyltransferase cst-II	*Campylobacter jejuni ATCC 43446*	2.4.99.-	AF167344	AAF34137.1		
α-2,3-sialyltransferase (Cst-II)	*Campylobacter jejuni ATCC 43456*	2.4.99.-	AF401526	AAL06000.1	Q93D05	
α-2,3-/α-2,8-sialyltransferase (CstII)	*Campylobacter jejuni ATCC 43460*	2.4.99.-	AY044868	AAK96001.1	Q938X6	
α-2,3/8-sialyltransferase (Cst-II)	*Campylobacter jejuni ATCC 700297*	n.d.	AF216647	AAL36452.1		
ORF	*Campylobacter jejuni GB11*	n.d.	AY422197	AAR82675.1		
α-2,3-sialyltransferase cstIII	*Campylobacter jejuni MSC57360*	2.4.99.-	AF195055	AAG29922.1		
α-2,3-sialyltransferase cstIII C|1140	*Campylobacter jejuni NCTC 11168*	2.4.99.-	AL139077 NC_002163	CAB73395.1 NP_282288.1	Q9PNF4	
α-2,3/α-2,8-sialyltransferase II (cstII)	*Campylobacter jejuni O:10*	n.d.	- AX934427	AAO96669.1 CAF04167.1		
α-2,3/α-2,8-sialyltransferase II (CstII)	*Campylobacter jejuni O:19*	n.d.	AX934431	CAF04169.1		
α-2,3/α-2,8-sialyltransferase II (CstII)	*Campylobacter jejuni O:36*	n.d.	AX934436	CAF04171.1		
α-2,3/α-2,8-	*Campylobacter*	n.d.	AX934434	CAF04170.1		

FIG. 2N

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / 3D
sialyltransferase II (CstII)	*jejuni O:4*					
α-2,3/α-2,8-sialyltransferase II (CstII)	*Campylobacter jejuni O:41*	n.d.	- - AX934429	AAO96670.1 AAT17967.1 CAF04168.1		
α-2,3-sialyltransferase cst-I	*Campylobacter jejuni OH4384*	2.4.99.-	AF130466 -	AAF13495.1 AAS36261.1	Q9RGF1	
bifunctional α-2,3/-2,8-sialyltransferase (Cst-II)	*Campylobacter jejuni OH4384*	2.4.99.-	AF130984 AX934425	AAF31771.1 CAF04166.1	1RO7 1RO8	C A
HI0352 (fragment)	*Haemophilus influenzae Rd*	n.d.	U32720 X57315 NC_000907	AAC22013.1 CAA40567.1 NP_438516.1	P24324	
PM1174	*Pasteurella multocida PM70*	n.d.	AE006157 NC_002663	AAK03258.1 NP_246111.1	Q9CLP3	
Sequence 10 from patent US 6503744	*Unknown.*	n.d.	-	AAO96672.1		
Sequence 10 from patent US 6699705	*Unknown.*	n.d.	-	AAT17969.1		
Sequence 12 from patent US 6699705	*Unknown.*	n.d.	-	AAT17970.1		
Sequence 2 from patent US 6709834	*Unknown.*	n.d.	-	AAT23232.1		
Sequence 3 from patent US 6503744	*Unknown.*	n.d.	-	AAO96668.1		
Sequence 3 from patent US 6699705	*Unknown.*	n.d.	-	AAT17965.1		
Sequence 34 from patent US 6503744	*Unknown.*	n.d.	-	AAO96684.1		
Sequence 35 from patent US 6503744 (fragment)	*Unknown.*	n.d.	- -	AAO96685.1 AAS36262.1		
Sequence 48 from patent US 6699705	*Unknown.*	n.d.	-	AAT17988.1		
Sequence 5 from patent US 6699705	*Unknown.*	n.d.	-	AAT17966.1		
Sequence 9 from patent US 6503744	*Unknown.*	n.d.	-	AAO96671.1		

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DeFrees, Shawn

<120> REMODELING AND GLYCOPEGYLATION OF FIBROBLAST GROWTH FACTOR (FGF)

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<140> N/A
<141> 2005-10-31

<150> US 60/623,342
<151> 2004-10-29

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Arg Pro Pro Leu Leu Gly Glu Arg Arg Ser Ala Ala Glu Arg Ser Ala
35 40 45

Arg Gly Gly Pro Gly Ala Ala Gln Leu Ala His Leu His Gly Ile Leu
50 55 60

Arg Arg Arg Gln Leu Tyr Cys Arg Thr Gly Phe His Leu Gln Ile Leu
65 70 75 80

Pro Asp Gly Ser Val Gln Gly Thr Arg Gln Asp His Ser Leu Phe Gly
85 90 95

Ile Leu Glu Phe Ile Ser Val Ala Val Gly Leu Val Ser Ile Arg Gly
100 105 110

Val Asp Ser Gly Leu Tyr Leu Gly Met Asn Asp Lys Gly Glu Leu Tyr
115 120 125

Gly Ser Glu Lys Leu Thr Ser Glu Cys Ile Phe Arg Glu Gln Phe Glu
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Glu Asn Trp Tyr Asn Thr Tyr Ser Ser Asn Ile Tyr Lys His Gly Asp
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Thr Gly Arg Arg Tyr Phe Val Ala Leu Asn Lys Asp Gly Thr Pro Arg
165 170 175

Asp Gly Ala Arg Ser Lys Arg His Gln Lys Phe Thr His Phe Leu Pro
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Pro Gly Ala Ala Gln Leu Ala
1 5

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Arg Ser Ala Thr Glu Thr Pro
1 5

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Arg Ser Gly Thr Glu Thr Pro
1 5

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Arg Ser Gly Thr Glu Thr Pro
1 5

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Arg Val Gly Thr Glu Thr Pro
1 5

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Gly Val Gly Thr Glu Thr Pro
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Gly Ser Ala Thr Glu Thr Pro
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Gly Val Gly Val Thr Glu Thr Pro
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Gly Val Thr Glu Thr Pro
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Gln Thr Glu Leu Pro
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Gly Val Thr Ser Ala Pro
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Ser Val Val Thr Pro
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Pro Thr Gly Ala Gln Leu Ala
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Pro Thr Gln Gly Ala Met Pro
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Pro Thr Gln Gly Ala Met Ala
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Pro Thr Thr Ala Gln Leu Ala
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Pro Gly Ala Thr Gln Leu Ala
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Pro Thr Ser Ser Ser Thr Ala
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<400> 65

Pro Thr Ser Ser Ser Leu Ala
1 5

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His Leu Gln Ile Leu Pro
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Pro Asp Gly Ser Val Gln Gly Thr
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Pro Asn Gly Ser
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Gln Thr Glu Leu Pro
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Leu Ile Val Thr Pro
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Leu Thr Glu Leu Pro
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Leu Thr Glu Leu Pro
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Gly Val Thr Ser Ala Pro
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His Leu Thr Glu Thr Pro
1 5

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Val Leu Thr Glu Thr Pro
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Val Gly Thr Glu Thr Pro
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Val Gly Val Gly Thr Glu Thr Pro
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Val Thr Ser Ala Pro
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Val Ser Thr Pro
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Glu Ala Thr Pro
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Pro Thr Gly Ser Val Gly Thr
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Pro Thr Gln Gly Val Gln Gly Thr
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Pro Thr Gly Ser Val Gly Pro Gly Thr
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Pro Thr Gln Gly Ala Met Pro Gly Thr
1 5

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Pro Thr Thr Ser Val Gln Gly Thr
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Pro Thr Thr Ala Val Gln Gly Thr
1 5

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Ile Leu Pro Asn Gly Ser Val His
1 5

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Ile Phe Pro Asn Gly Ser Val
1 5

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Pro Asn Gly Thr
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<400> 90

Leu Pro Asn Gly Thr Val His
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Pro Asn Gly Thr Val
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Ile Leu Pro Asn Gly Thr
1 5

<210> 93
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<400> 93

Gln Ile Leu Pro Asn Gly Thr
1 5

<210> 94
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<400> 94

Gln Ile Leu Pro Asn Gly Thr Val His
1 5

<210> 95
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<400> 95

Leu Asn Val Thr Glu Thr Pro Arg Asp Gly Ala Arg Ser Lys Arg His
1 5 10 15

<210> 96
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<400> 96

Leu Asn Val Thr Glu Thr Pro Asp Asp Gly Ala Arg Ser Lys Arg His
1 5 10 15

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<400> 97

Leu Asn Val Thr Glu Thr Pro Leu Asp Gly Ala Arg Ser Lys Arg His
1 5 10 15

<210> 98
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<220>
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<400> 98

Leu Asn Ala Ile Thr Thr Pro Arg Asp Gly Ala Arg Ser Lys Arg His
1 5 10 15

<210> 99
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<220>
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<400> 99

Leu Asn Ala Ile Thr Thr Pro Leu Asp Gly Ala Arg Ser Lys Arg His
1 5 10 15

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<400> 100

Leu Asn Gln Glu Ala Thr Pro Leu Asp Gly Ala Arg Ser Lys Arg His
1 5 10 15

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<400> 101

Leu Asn Gln Thr Glu Leu Pro Leu Asp Gly Ala Arg Ser Lys Arg His
1 5 10 15

<210> 102
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<400> 102

Leu Asn Gln Thr Glu Leu Pro Ala Asp Gly Ala Arg Ser Lys Arg His
1 5 10 15

<210> 103
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<400> 103

Leu Asn Lys Asp Gly Thr Pro Thr Asp Gly Ala Arg Ser Lys Arg His
1 5 10 15

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<400> 104

Leu Asn Lys Asp Gly Thr Pro Thr Ser Gly Ala Arg Ser Lys Arg His
1 5 10 15

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<400> 105

Leu Asn Lys Asp Gly Thr Pro Thr Asp Gly Ala Ala Ser Lys Arg His
1 5 10 15

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<400> 106

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Leu Asn Lys Asp Gly Thr Pro Thr Ser Gly Ala Ala Ser Lys Arg His
1 5 10 15

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<400> 107

Leu Asn Lys Asp Gly Thr Pro Thr Gln Gly Ala Met Pro Lys Arg His
1 5 10 15

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<400> 108

Leu Asn Lys Asp Gly Thr Pro Thr Gln Gly Ala Met Ser Lys Arg His
1 5 10 15

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<220>
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<400> 109

Leu Asn Lys Asp Gly Thr Pro Thr Thr Thr Ala Arg Ser Lys Arg His
1 5 10 15

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<400> 110

Leu Pro Arg Pro Val Asp Pro Glu Arg Val Pro Glu Leu Tyr Lys Asp
1 5 10 15

<210> 111
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<400> 111

Pro Glu Arg Val Pro Glu Leu Tyr Lys Asp
1 5 10

<210> 112
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<220>
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<400> 112

Leu Pro Ala Pro Thr Asp Pro Glu Arg Val Pro Glu Leu Tyr Lys Asp
1 5 10 15

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<220>
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<400> 113

Leu Pro Asn Pro Thr Ala Pro Glu Arg Val Pro Glu Leu Tyr Lys Asp
1 5 10 15

<210> 114
<211> 16
<212> PRT
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<220>
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<400> 114

Leu Pro Arg Pro Thr Ala Pro Glu Arg Val Pro Glu Leu Tyr Lys Asp
1 5 10 15

<210> 115
<211> 16
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<220>
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<400> 115

Leu Pro Ala Pro Thr Gln Pro Glu Arg Val Pro Glu Leu Tyr Lys Asp
1 5 10 15

<210> 116
<211> 16
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<220>

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<400> 116

Leu Pro Thr Pro Val Asp Pro Glu Arg Val Pro Glu Leu Tyr Lys Asp
1 5 10 15

<210> 117
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<220>
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<400> 117

Leu Pro Thr Pro Ser Asp Pro Glu Arg Val Pro Glu Leu Tyr Lys Asp
1 5 10 15

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<400> 118

Leu Pro Val Pro Thr Asp Pro Glu Arg Val Pro Glu Leu Tyr Lys Asp
1 5 10 15

<210> 119
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<220>
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<400> 119

Leu Pro Thr Pro Ala Asp Pro Glu Arg Val Pro Glu Leu Tyr Lys Asp
1 5 10 15

<210> 120
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<220>
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<400> 120

Pro Thr Ala Ser Pro Glu Leu Tyr Lys Asp
1 5 10

<210> 121
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<220>
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<400> 121

Pro Thr Ala Ser Pro Ala Leu Tyr Lys Asp
1 5 10

<210> 122
<211> 10
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<220>
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<400> 122

Pro Asn Thr Leu Pro Glu Leu Tyr Lys Asp
1 5 10

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<400> 123

Pro Glu Thr Val Pro Glu Leu Tyr Lys Asp
1 5 10

<210> 124
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<220>
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<400> 124

Pro Gln Glu Thr Pro Glu Leu Tyr Lys Asp
1 5 10

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<220>
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<400> 125

Pro Thr Gln Gly Pro Glu Leu Tyr Lys Asp
1 5 10

<210> 126
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<212> PRT
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<400> 126

Pro Thr Gln Gly Pro Ala Leu Tyr Lys Asp
1 5 10

<210> 127
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<400> 127

Pro Gln Gly Thr Pro Ala Leu Tyr Lys Asp
1 5 10

<210> 128
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<400> 128

Pro Ala Thr Glu Pro Glu Leu Tyr Lys Asp
1 5 10

<210> 129
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<400> 129

Pro Thr Thr Gln Pro Glu Leu Tyr Lys Asp
1 5 10

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<400> 130

Pro Thr Thr Glu Pro Glu Leu Tyr Lys Asp
1 5 10

<210> 131
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<400> 131

Pro Glu Arg Val Pro Thr Leu Tyr Lys Asp
1 5 10

<210> 132
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<400> 132

Pro Glu Arg Val Pro Thr Leu Tyr Ala Asp
1 5 10

<210> 133
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<400> 133

Pro Glu Arg Val Pro Thr Gln Gly Ala Asp
1 5 10

<210> 134
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<213> Artificial Sequence

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<400> 134

Pro Glu Arg Val Pro Thr Gln Gly Ala Met Pro
1 5 10

<210> 135
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<220>
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<400> 135

Pro Glu Arg Val Pro Thr Gln Gly Ala
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<210> 136
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<220>
<221> misc_feature
<222> (6)..(6)
<223> Xaa can be any naturally occurring amino acid

<400> 136

Leu Met Tyr Thr Pro Xaa
1 5

<210> 137
<211> 6
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<220>
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<220>
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<223> Xaa can be any naturally occurring amino acid

<400> 137

Leu Thr Glu Thr Pro Xaa
1 5

<210> 138
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<220>
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<222> (6)..(6)
<223> Xaa can be any naturally occurring amino acid

<400> 138

Val Thr Glu Thr Pro Xaa
1 5

<210> 139
<211> 8
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<220>
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<220>
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<223> Xaa can be any naturally occurring amino acid

<400> 139

Gly Val Thr Glu Thr Pro Leu Xaa
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<210> 140
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<223> X is selected from OH, NH2, glycine, alanine, leucine, and asparagine

<220>
<221> misc_feature
<222> (13)..(13)
<223> Xaa can be any naturally occurring amino acid

<400> 140

Pro Glu Leu Tyr Val Gly Val Thr Cys Thr Pro Leu Xaa
1 5 10

<210> 141
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<220>
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<223> X is selected from OH, NH2, glycine, alanine, leucine, and asparagine

<220>
<221> misc_feature
<222> (5)..(5)
<223> Xaa can be any naturally occurring amino acid

<400> 141

Leu Met Tyr Thr Xaa
1 5

<210> 142
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<212> PRT
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<220>
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<400> 142

Leu Met Tyr Thr Pro Thr Ala Ser Pro
1 5

<210> 143
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<400> 143

Leu Met Tyr Thr Pro Ala Thr Glu Pro
1 5

<210> 144
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<220>
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<222> (8)..(8)
<223> Xaa can be any naturally occurring amino acid

<400> 144

Leu Met Tyr Thr Pro Thr Pro Xaa
1 5

<210> 145
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<222> (9)..(9)
<223> Xaa can be any naturally occurring amino acid

<400> 145

Leu Met Tyr Thr Pro Thr Ala Pro Xaa
1 5

<210> 146
<211> 182
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<213> Homo Sapiens

<400> 146

Met His Pro Ile Pro Asp Ser Ser Pro Leu Leu Gln Phe Gly Gly Gln
1 5 10 15

Val Arg Gln Arg Tyr Leu Tyr Thr Asp Asp Ala Gln Gln Thr Glu Ala
20 25 30

His Leu Glu Ile Arg Glu Asp Gly Thr Val Gly Gly Ala Ala Asp Gln
35 40 45

Ser Pro Glu Ser Leu Leu Gln Leu Lys Ala Leu Lys Pro Gly Val Ile
50 55 60

Gln Ile Leu Gly Val Lys Thr Ser Arg Phe Leu Cys Gln Arg Pro Asp
65 70 75 80

Gly Ala Leu Tyr Gly Ser Leu His Phe Asp Pro Glu Ala Cys Ser Phe
85 90 95

Arg Glu Leu Leu Leu Glu Asp Gly Tyr Asn Val Tyr Gln Ser Glu Ala
100 105 110

His Gly Leu Pro Leu His Leu Pro Gly Asn Lys Ser Pro His Arg Asp
115 120 125

Pro Ala Pro Arg Gly Pro Ala Arg Phe Leu Pro Leu Pro Gly Leu Pro
130 135 140

Pro Ala Leu Pro Glu Pro Pro Gly Ile Leu Ala Pro Gln Pro Pro Asp
145 150 155 160

Val Gly Ser Ser Asp Pro Leu Ser Met Val Gly Pro Ser Gln Gly Arg
165 170 175

Ser Pro Ser Tyr Ala Ser
180

<210> 147
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<212> PRT
<213> Artificial Sequence

<220>
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<400> 147

Met His Pro Ile Pro Asp Ser Ser
1 5

<210> 148
<211> 5
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<213> Artificial Sequence

<220>
<223> FGF mutation motif

<400> 148

Pro Leu Leu Gln Phe
1 5

<210> 149
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<213> Artificial Sequence

<220>
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<400> 149

Ala Asp Gln Ser Pro Glu Ser Leu Leu
1 5

<210> 150
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<220>
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<400> 150

Lys Pro Gly Val Ile Gln
1 5

<210> 151
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
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<400> 151

Arg Pro Asp Gly Ala Leu
1 5

<210> 152
<211> 6
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<220>
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<400> 152

Ser Leu His Phe Asp Pro
1 5

<210> 153
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<220>
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<400> 153

Ala His Gly Leu Pro Leu His Leu Pro Gly Asn Lys Ser Pro His Arg
1 5 10 15

Asp Pro Ala Pro Arg Gly Pro Ala Arg Phe Leu Pro Leu Pro
20 25 30

<210> 154
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<212> PRT
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<220>
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<400> 154

Glu Pro Pro Gly Ile Leu Ala Pro Gln
1 5

<210> 155
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<220>
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<400> 155

Asp Val Gly Ser Ser Asp Pro
1 5

<210> 156
<211> 6
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<220>
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<400> 156

Leu Ser Met Val Gly Pro
1 5

<210> 157
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<212> PRT
<213> Artificial Sequence

<220>
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<400> 157

Ser Gln Gly Arg Ser Pro Ser Tyr Ala Ser
1 5 10

<210> 158
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<400> 158

Met His Pro
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<210> 159
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<220>
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<400> 159

Met His Pro Ile Pro
1 5

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Ser Leu Thr Glu Thr Pro

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Thr Ser Gly Ala Met Pro
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Ser Gln Gly Ala Ser Pro
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Ala Ser Pro Thr Ile Asn Thr Pro
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Pro Pro Thr Gln Ala Leu Pro Pro
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Pro Pro Thr Thr Val Ser Arg Arg Ser

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Pro Thr Thr Val Ser Leu Ala
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Pro Thr Gln Ala Gln Leu Ala
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Pro Thr Ile Asn Thr Gln Gly Thr
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Pro Thr Thr Val Ser Gln Gly Thr
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Leu Asn Lys Asp Gly Thr Pro Thr Ile Asn Thr Ser Ser Lys Arg His
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